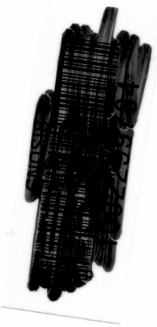

A TOXICITY ASSESSMENT OF TOTAL DISSOLVED SOLID IONS IN MINE EFFLUENT USING TWO COMMON BIOASSAYS: THE 22-HOUR MICROTOX® ASSAY AND A *S.CARPRICORNUTUM* GROWTH ASSAY

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A THESIS

Presented to the Faculty of the University of Alaska Fairbanks in Partial Fulfillment of the Requirements for the Degree of

MASTER OF SCIENCE

By

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Fairbanks, Alaska

May 2000

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Abstract

This research evaluated two microassays and a synthetic TDS standard to measure the effects of elevated TDS from mine effluent on biota of freshwater systems. Field samples from Red Dog and Fort Knox Mines were tested on *Selenastrum capricornutum* and the MircoTox® assay, and compared to the synthetic standard. Results indicate that the synthetic TDS standard is a poor representation of produced waters with similar total TDS concentrations. Additionally, no correlation was found between the toxicological responses of the two assays. Principle component analysis found the MicroTox® assay to be most sensitive to chloride and ammonia, while the *S. capricorutnum* assay is most sensitive to cadmium and chloride. At concentrations present in the field samples, there does not appear to be a relationship between toxicity and TDS as measured on these assays.

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List of Abbreviations

ADEC	Alaska Department of Environmental Conservation
AGP	Algae Growth Potential
APHA	American Public Health Association
ASTM	American Society for Testing and Materials
CIP	Carbon In Pulp
ECxx	Effective Concentration for xx percent response
ICP	Inductively Coupled Plasma
IW	Interceptor Well
LDxx	Lethal Dose for xx Percent
MW	Monitoring Well
NTAC	National Technological Advisory Committee
PC	Principle Component
QC	Quality Control
RO	Reverse Osmosis
TDS	Total Dissolved Solids
USEPA	United States Environmental Protection Agency

Chapter 1

Introduction

Within state borders, Alaska encompasses over 365,000 miles of freshwater rivers and streams (ADEC, 1986). Like the surrounding land, these streams provide a wealth of natural resources that support the State's economy. Mining, fishing, and tourism are three of the top five principle industries of Alaska, (Famighetti, 1994) and are all, at least in part, dependent upon an extensive network of freshwater rivers and streams. Due to the shared nature of these delicate ecosystems, regulations have been implemented to ensure the survival of each industry, as well as the natural integrity of wilderness areas.

One industry that can have a large effect on the ecosystem is hard rock mining. In 1985, 5.4 million grams of gold were recovered from gold mines, generating $62 million in revenue (ADEC, 1986). Unfortunately, if not well managed, the environmental cost from these activities can be tremendous contamination. Contamination can originate from both point sources, such as effluent releases and tailing ponds, and non-point sources, including overburden, runoff, and road erosion (ADEC, 1986). Much of this rock waste, which can include acid generating ores, heavy metals, dissolved materials, and sediment, can be stored at the mine site, or treated and released into neighboring streams, "receiving waters". In 1986, an estimated 2000 miles of freshwater rivers and streams were contaminated as a result of surface activities (ADEC, 1986). Many of these mines are located in the interior of the State where gold ore is most abundant.

Figure 1. Mining Sites and Prospects in Alaska (www.commerce.state.ak.us/trade/mines_ma.htm)

Since 1976, under the guidance of the EPA, Alaska has been active in establishing State standards and industrial regulations for the release of toxins and contaminants that pose a threat to aquatic life. Many of these are similar to national levels, but some have required further study to suit unique Alaskan conditions. One regulation under continued

re-examination is that for total dissolved solids, TDS. Because of variable background levels, inconsistent definitions, and multiple methods of measure, TDS criteria have come under reconsideration by the State of Alaska. Currently, there are no federal regulations regarding TDS. The Alaska Department of Environmental Conservation recently revised the water quality criterion for TDS in 1999. As of this time, released water is not to exceed 500mg/L TDS. Conditional permits may be granted for the release of water with TDS levels between 500 and 1000mg/L if it is proven by the permit seeker that the proposed TDS level will not cause, or be expected to cause, an adverse effect to aquatic life (ADEC, 1999). This criterion has not been accepted by the USEPA, but is expected to be reviewed by the agency in the summer of 2000.

The primary objective of this project was to develop a method capable of showing the presence of adverse effects to aquatic life at TDS levels between 500 and 1000mg/L. This involved an investigation of two common bioassays, and their responses to elevated levels of TDS. The assay study included a comparison between sample results, method sensitivity, and method precision. Principle component analysis was used to characterize the specific sensitivity of each assay to particular ionic components. Further work with field samples included EC_{20} measurements, determination of sample stability during storage, and receiving water dilution effects.

Second, this work evaluated a synthetic TDS solution with an ion content characteristic of hard rock mine effluent. Monthly samples from several sites around the Fort Knox Gold Mine and the Red Dog Mine were collected, and tested using the MicroTox® assay and a *Selenastrum capricornutum* growth assay. EC_{20} results from

these samples were compared to the toxicity measurements of the synthetic TDS solution. These results were then modeled, using principle component analysis, and compared.

1.1 Total Dissolved Solids

A rigorous definition of TDS has not been universally accepted among environmental agencies. The American Public Health Association (APHA) defines TDS as the solid residue left in a vessel after the evaporation and drying of a filtered sample, at a prescribed temperature. This residue may include inorganic salts, organic acids, and other dissolved materials (APHA, 1995). However, several Western states including Wyoming and Colorado have specifically defined TDS as being synonymous with salinity (USEPA, 1980), and frequently EPA documents use total dissolved solids and salinity interchangeably (USEPA, 1977). In doing so, TDS becomes defined as "the principal inorganic anions and cations dissolved in water: the carbonates, chloride, sulfate, nitrate, sodium, potassium, calcium, and magnesium" (USEPA, 1976). Research regarding production water effluent conducted by ENSR and the Gas Research Institute, found that the predominant contaminants of produced water were dissolved salts. The primary ion species again included the carbonates, chloride, sulfate, nitrate, sodium, potassium, calcium, and magnesium (Mount et al., 1992). Although the APHA does not recognize ion concentration as a precise measure of TDS, current manuals use ion concentration measurements to approximate total dissolved solid levels (APHA, 1995). This study is specifically concerned with the behavior of tailings pond effluent. Concentrations of the ions listed above are used to estimate the TDS levels of freshwater systems in question.

All freshwater has a naturally occurring level of TDS, which is primarily a function of the rock and sediment composition of the stream channel and surrounding drainage area. Due to the immensely varying geographical and geological conditions of the State, these natural levels can differ considerably. Measurements have been made ranging from 19 ppm to over 64,000 ppm in mineral springs. Most, however, are less than 250 ppm (Balding, 1976). Generally, waters with TDS concentrations less than 1000 ppm are considered freshwater, those between 1000 and 15,000 are brackish, and concentrations over 15,000 ppm constitute saline waters (Water Quality Association, 1999). With such varying background levels, environmental agencies have encountered difficulty in establishing Statewide regulations that are applicable to all systems.

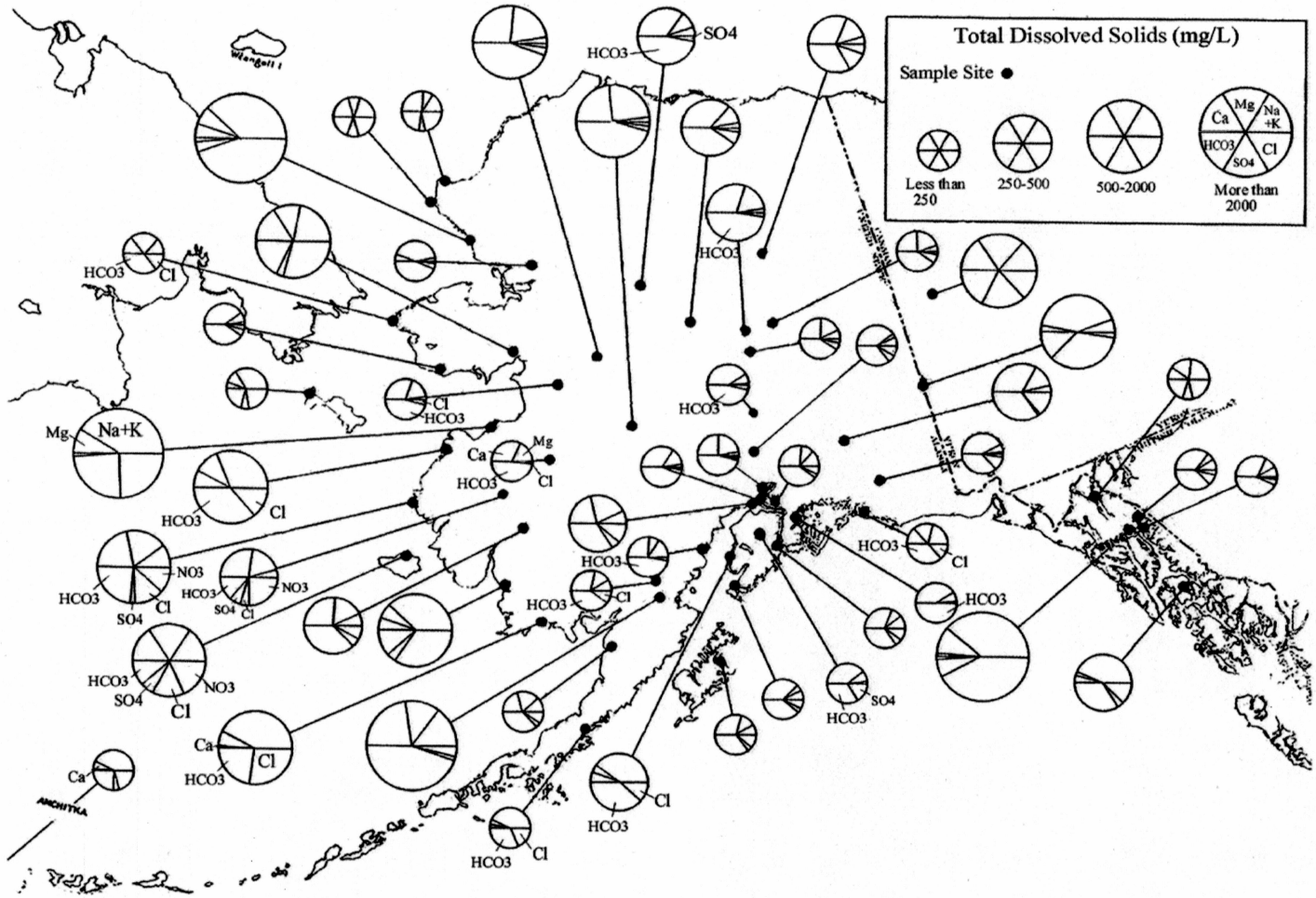


Figure 2. Naturally occuring levels of TDS in freshwater systems throughout Alaska (Baulding, 1976)

Due in part to its inconsistent definition, there are many methods of measuring TDS. The standard method of the APHA is the most common laboratory procedure. A sample is filtered through a glass fiber filter into a crucible, then dried at 180° C for at least one hour. The mass of the residue is taken as the TDS per volume of sample water used, and is expressed as mg/L TDS. Several minor limitations are associated with this method. In drying the sample, a residue seal may form, preventing total evaporation of the sample filtrate (Brown, 1970). Even at high temperatures, calcium sulfate salts tend to retain the water of crystallization (Brown, 1970). Water high in nitrate may lose up to 30 mg/L due to volatilization (Brown, 1970). Finally, some calcium and magnesium chloride may also be lost to volatilization (Brown, 1970).

Conductivity, traditionally used to measure salinity, can be used for estimating TDS. This method is not as precise as the APHA's procedure for evaluating total dissolved material, but can be more specific in its measure of ion content in a sample. Conductivity is dependent upon the ionic concentration, mobility, valence, and sample temperature (APHA, 1995). To convert to TDS values, conductivity is multiplied by an empirical factor, which is dependent on the temperature and ion species present in the sample water (APHA, 1995). This procedure is frequently used when general values are acceptable because it can be done easily in the field. When translating to TDS measurements, though, this method is susceptible to a fundamental error. Dissolved inorganic compounds are usually good conductors, but organic molecules that do not dissociate will not carry an electrical current (APHA, 1995). The result can be an underestimation of total dissolved material in solution. Hence, when defining TDS to

include dissolved organic and inorganic matter, this method should be used for approximations only.

1.2 TDS and Fish Populations

Many metals and minor constituents can be toxic to aquatic organisms in trace quantities. For many ionic species, the resulting effects on biota of freshwater systems is greatly dependent upon the valence configuration of the ion and the pH of the solution. For example, chromium (VI), in aqueous systems as dichromate or chromate anions, is more toxic and more readily absorbed than Cr (III) (Tiesser and Turner, 1995). Table 1 includes a partial list of components that can be lethal to freshwater fishes and other organisms at low concentrations.

Table 1. Toxicity of trace components in freshwater systems

Component	Toxicity concentration	Method	Species	Source
Al	0.12 mg/L	96h LC50	Rainbow Trout	Holtz, 1983
Ag	7.6-10.9 μg/L	96h LC50	Rainbow Trout	EPA, 1980b
As	23-26.6 mg/L	96h LC50	Rainbow Trout	Spehar, 1980
Cd	6.6 μg/L	96h LC50	Rainbow Trout	Hale, 1977
Cr (VI)	30.5 mg/L	96h LC50	Rainbow Trout	Krishnja and Rege, 1982
Cu	110 μg/L	96h LC50	Rainbow Trout	Birge, 1979
Hg	5.0-42.0 μg/L	96h LC50	Rainbow Trout	EPA, 1980a
NH_4	0.16-1.1 mg/L	96h LC50	Rainbow Trout	Calamari, 1977, 1981; Broderius and Smith, 1979; DeGraere, et al., 1980; Thurston, Philips et al., 1981; Thurston, Russo, et al.,1981; Reinbold and Pescitelli, 1982; West, 1985; Thurston and Russo, 1983
NO_3	6.0 g/L	96h LC50	Rainbow Trout	Westin, 1974
NO_2	0.19-0.39 mg/L	96h LC50	Rainbow Trout	Russo, 1974
Pb	1.20 mg/L	96h LC50	Rainbow Trout	Demayo, 1980
Se	8.0 mg/L	96h LC50	Rainbow Trout	Hodsen, 1984
Zn	0.9-7.21 mg/L	96h LC50	Rainbow Trout	EPA, 1980

Despite the toxic effects of some components, many of the dissolved materials found in freshwater systems are necessary for the growth and survival of aquatic organisms. When present in correct stoichiometric proportions, cations, anions, and trace metals can create a favorable environment for fish and other aquatic species (USEPA, 1977).

When compared to the toxic effects of several metals and other ionic chemical species such as ammonia and nitrite, total dissolved solid salts appear rather benign. In fact, some ions species may even protect freshwater organisms from the toxic effects of dissolved metals. For example, sulfate has been shown to lessen the toxic effects of chromium in several species of freshwater algae (Riedel, 1989). Another study found that heavy metals are rendered less toxic in harder waters, especially those that contain elevated levels of calcium and magnesium (Rand and Perrocelli, 1985). This same study, however, found that salinity does not have a great modifying effect on the toxicity of other pollutants (Rand and Perrocelli, 1985).

Elevated levels of TDS can have detrimental effects on freshwater biota, however, including increased osmotic stress and other physiological disturbances (USEPA, 1977). According to Brown (1970), freshwater fish have osmotic blood pressures equal to six atmospheres, or 7000 mg/L NaCl. Thus, for practical purposes, water with ion concentrations of greater than 7000 mg/L can be lethal.

Aside from ion pressure effects, elevated TDS levels also affect uptake of nutrients and dissolved oxygen. Rao (1996) found that oxygen consumption for rainbow trout was lowest for those raised in saline water solutions. Reproduction and survival can also be affected. Successful fertilization and maturation of minnow species in freshwater streams have been documented to be significantly reduced due to TDS increases within a system (Boelter et al., 1992). This may occur when effluent discharge into receiving streams increases, or when the receiving water levels decrease due to drought or seasonal

variations in flow. In her study, Boelter (1992) also ascertained that invertebrates and fry had a greater sensitivity to these dissolved contaminates than did adult fish.

Despite some recommended limits, all species of fish and aquatic life need to be able to adapt to a range of dissolved solid concentrations to survive in dynamic natural conditions. Studies in Canada by Rawson and Moore (1944) found that a number of freshwater species of fish are able to survive concentrations of 10,000 mg/L dissolved solids, but that only whitefish and pikeperch are able to withstand levels as high as 15,000 mg/L. In addition to the direct effects on fish, habitat-forming plants are also damaged when TDS levels are high. The National Technical Advisory Committee, NTAC, recommends that in order to protect fish populations, no change in stream hydrography or flow should be allowed that causes lasting changes of natural isohaline patterns by more than 10% (USEPA, 1976).

The dangers of TDS to aquatic organisms can be two fold. In addition to the cumulative effects of dissolved solids, the ionic composition of these dissolved solids can have profound consequence on the biota of the receiving water. While some ions may have protective effects against trace components, freshwater species are selectively sensitive to others. Of the major ion species tested in previous studies, including chloride, sodium, potassium, magnesium, sulfate, and calcium, chloride appears to be the most toxic to freshwater organisms (Mount et al., 1992). When trying to predict the effects of released effluent on receiving waters, ionic composition, as well as total dissolved solid concentrations must be considered.

1.3 TDS and Toxicity Analysis

TDS ions are considered secondary contaminents by the USEPA. Measuring and monitoring dissolved ion toxicity in effluent discharge is important for protecting biota of the receiving water. In addition, the toxicity of total dissolved solids is an important factor for predicting how the effluent can best be treated. Ionic pollutants are not treated by traditional wastewater treatments such as aeration (Gulley et al., 1992). Instead, dilution, precipitation by calcium hydroxide, and reverse osmosis treatments are most often used to lessen toxic effects on the environment.

Most industrial effluent tends to be much higher in dissolved material than the receiving freshwater (O'Neil, 1992), although composition can vary significantly depending on the type of industry, purpose of water in operations, and geographical site. Furthermore, synergistic and antagonistic interactions may occur between ion species. As a result, an effluent's TDS concentration may not accurately reflect its toxic effects on the aquatic community (Mount et al., 1992).

Instead of ion specific research, past reports indicate that a more useful approach is one that addresses the toxicity of the complex ion mixture present in effluent discharge (Mount and Gulley, 1992). Changes made to USEPA policy during the 1980's has led to the increased use of "whole effluent testing, or bio-monitoring" for the regulation of effluent discharge to surface waters (Mount and Norberg, 1984).

Because of the variability of effluent, and the dissimilar reactions of aquatic biota, bioassay testing may give the most accurate response of an aquatic community to an effluent discharge. In a 1992 report on the toxicity of saline water on freshwater

organisms, authors Mount and Gulley (1992) argue that even when analytical and toxicological data are available, toxic interactions among mixtures cannot be predicted. They concluded, "The most direct and cost-effective indication of whole effluent toxicity is to conduct actual toxicity tests with aquatic organisms". Therefore, the most conclusive results of this study will be drawn from the laboratory findings and responses to the bioassay testing.

Conducting environmental studies on a microcosmic scale introduces several points of uncertainty, due in part to the elimination of many variables present in an organism's natural environment. These may include, and are not limited to, temperature fluctuations, food and light availability, disease, and predators. However, despite the confines of the laboratory, several past studies indicate that responses shown by aquatic organisms in the lab are remarkably similar to those exhibited in their natural habitat. In the same report by Mount and Gulley (1992), their results of effluent toxicity tests in the lab corresponded well with the results of further instream studies. Thus, using laboratory data has been proven to be a valid method for anticipating the survival of freshwater organisms, and for predicting the toxicity of major ions (1992).

1.4 Notation of Toxicity Measurements

Toxicity measurements are typically reported as a lethal concentration (LC) or an effective concentration (EC) at a designated percent value. The percent value refers to the percent of assay organisms which reach the predetermined endpoint during an experiment. LC is used when the endpoint is death of the organism, whereas an EC value

is reported when any other physiological endpoint is used. A commonly reported toxicity value is LC_{50}, which indicates the percent of a solution that is lethal to 50% of the assay organisms. It is important to note that low EC or LC values denote a highly sensitive species, while greater values indicate a species that is more tolerant of the tested toxicant.

1.5 Proposed Synthetic TDS Solution

Regulated industries in Alaska are interested in simplifying dissolved solid toxicity tests. Ideally, these industries would like to be able to predict the toxicity of effluent due to TDS levels, and demonstrate when elevated levels would have no detrimental effects on the aquiculture of the receiving water. Having a synthetic TDS standard to predict toxic effects of produced waters would be a tremendous advantage over testing water samples individually on expensive and time consuming fish and invertebrate assays. In order to do this, a model of the relationship between toxicity and TDS concentration needs to be constructed. Synthetic TDS solutions based on actual effluent concentrations have been suggested for this work.

Typical ore deposits in Alaska are sulfate deposits, consisting of metals in the form of MSO_4, where M is any metal cation. Tailings solutions must be treated to remove these metals before being released. This is done with calcium hydroxide, which precipitates metals as metal hydroxides, and produces solutions high in calcium and sulfate.

$$Ca(OH)_2 + M_2SO_4 \rightarrow 2MOH_{(s)} + Ca^{2+}{}_{(aq)} + SO_4^{2-}{}_{(aq)}$$

One synthetic solution that has been proposed is a 2500ppm solution of metal salts in distilled water. This solution includes the primary ions and cations that are included in TDS measurements, but does not contain any carbonate or nitrate species, as indicated in Table 2. These compounds, although present in significant concentrations in natural systems, are not appreciably present in mine effluent waters.

Omitting these ions from our synthetic TDS solution greatly simplifies the chemistry of the solution. The equilibrium of carbonate species in aqueous solutions is greatly dependent upon pH.

$$CO_2 + H_2O \rightleftharpoons H^+ + HCO_3^- \rightleftharpoons 2H^+ + CO_3^{2-}$$

The carbonate ion, CO_3^{2-}, is capable of complexing and removing some metals ions from solution by ligand binding. Thus, factors that may affect pH, such as exposure to air, temperature, and storage time, will have a great effect on the solution composition. These interactions can therefore alter the toxicity of a water sample, if the measured toxicity is due to the metal ions present. By not including these species in the solution, we have eliminated these effects.

Table 2. Ionic composition of synthetic TDS solution

Compound	g/L	Equivalent moles	Resulting Solution	mg/L (ppm)
$CaSO_4 \cdot 1/2H_2O$	2.3	0.01586	Ca^{2+}	648
$Na_2SO_4 \cdot 10H_2O$	0.3	0.00093	Na^+	38
$MgSO_4 \cdot 7H_2O$	0.4	0.00162	Mg^{2+}	40
KCl	0.03	0.00040	K^+	15
SO_4^{2-}	-	0.01842	SO_4^{2-}	1700
			Cl^-	14
			Total TDS	2500

1.6 Fort Knox Mine

Figure 3. Fort Knox Mine, Fox, Alaska.

Fort Knox Mine is an open pit gold mine located 15 miles northeast of Fairbanks, and is the largest gold producer in the State of Alaska. Construction on the mine began in 1995, and the first gold was poured in November 1996 (Szumigala and Swainbank,

1998). Since this time, Fort Knox Mine has steadily increased its production capacity to the current levels of 350,000 ounces per year.

Approximately 80,000 to 100,000 tons of rock are mined each day using standard drill and blast techniques. The mill then processes 36,000 to 50,000 tons of ore per day, and is capable of working 365 days a year. The mill has a 90% recovery rate, using a cyanidation/CIP method, which involves the use of cyanide to dissolve gold from the rock ore. In order to prevent the production of hydrogen cyanide gas, lime is added to the slurry to maintain a basic solution. Tailings are treated to remove cyanide before they are discharged to the impoundment (Szumigala and Swainbank, 1998). The gold reserve is estimated at 116 million grams in 161.8 million tons of ore with a grading of 0.8g/t.

Figure 4 shows the layout of the Fort Knox Mine site. The northwest area of the site houses the processing mill, which is surrounded by several rock dumps and soil stockpiles. The mill is located directly north of the open pit, and is also surrounded by rock and soil stockpiles. To the east of these sites is the tailings basin, a very large dammed basin surrounded by service roads. The rock dam lies approximately in the middle of the entire site. Directly east of the dam is a series of interceptor wells and pumps, which intercept any tailings water that seeps under the dam, and pumps it back to the basin. Farther east, of these interceptor wells is a series of monitoring wells, which are used to monitor the quality of groundwater flowing between the tailings pond and the freshwater reservoir. Between these pumps and the freshwater reservoir is an expanse of wetlands area that is undeveloped. At the easternmost end of the Fort Knox Mine property is the freshwater reservoir which is fed by a series of creeks, including Fish

Creek, Last Chance Creek, and Solo Creek. Bear Creek also flows into the reservoir by way of Fish Creek. There is a pump house at this site to pump freshwater to the mill and tailings facility when needed.

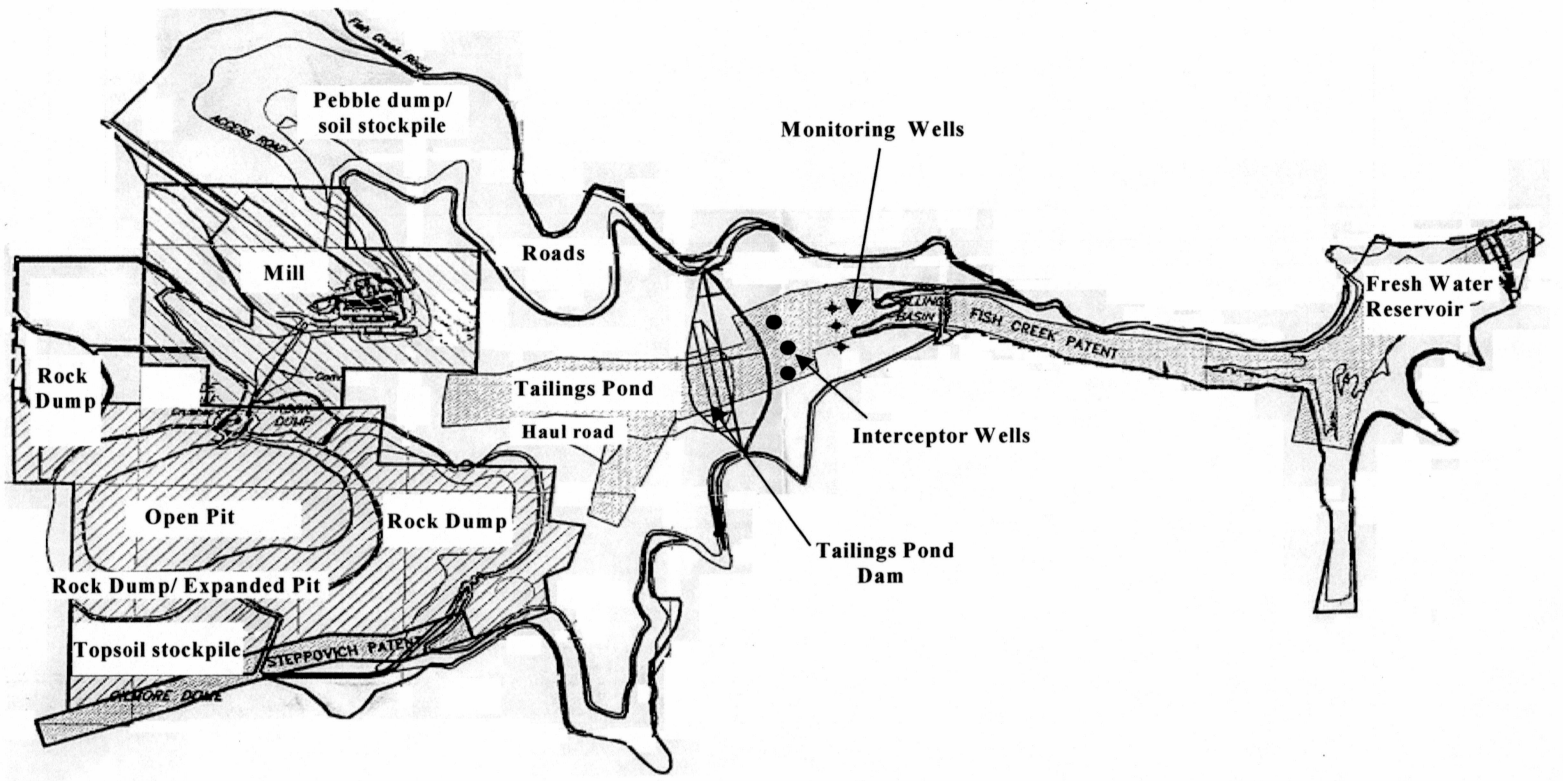


Figure 4. Fort Knox Mine operations site.

Water usage at the facility is conserved by treating water from the tailings pond and recycling it through the mill. Freshwater from the reservoir is used as a supplementary water supply only when needed.

1.7 Red Dog Mine

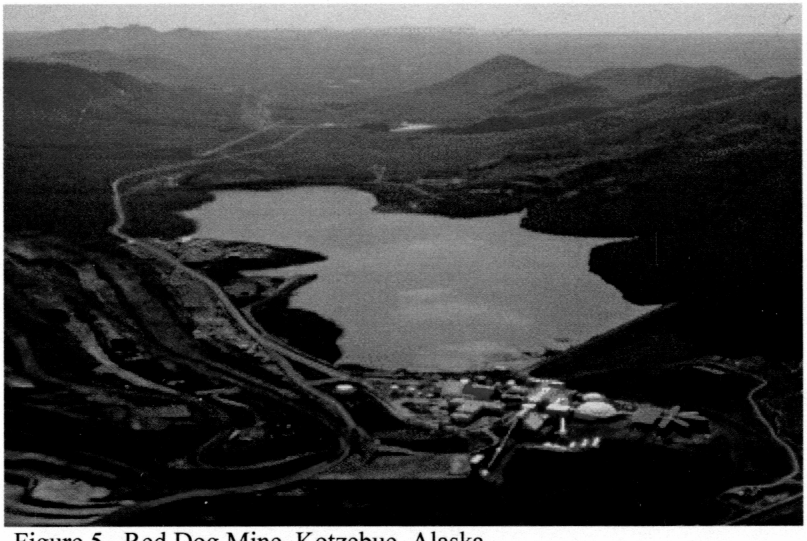


Figure 5. Red Dog Mine, Kotzebue, Alaska.
(www.cominco.com/ops/RedDog/sustublepres/sld007.htm).

Red Dog Mine is an open pit zinc, lead, and silver mine located 145 kilometers north of Kotzebue. It is the largest zinc producer in the world, generating 560,000 short tons of ore per year. The ore body itself is approximately 4400 feet long and 500 feet thick, averaging 21.4% zinc, 5.2% lead, and yielding 2.4 ounces of silver per short ton.

Construction on the Red Dog site began in 1986, and the ore mill began production in October 1989. Operations at the mine include blasting and milling of up to 2.7 million short tons of ore rock per year. The resulting concentrate is then hauled 52 miles to the port site on the Chukchi Sea, and shipped to other countries to be smelted.

Approximately 25% is shipped to Japan and Korea, 25% to Europe, and 50% to Canada.

The Red Dog valley contains numerous freshwater streams of varying quality. Most tend to be clear, with low levels of suspended solids and nutrients. Red Dog Creek, the site of the mining operation, consists of a Middle and a North Fork. The main diversion and drainage of the effluent is centered on the Middle Fork. The upper Middle Fork, and the entire North Fork are clean and clear. However, the mid and lower Middle Fork contains elevated concentrations of cadmium, lead, zinc, and iron, leached from the bedrock. As a result, the creek waters are high in total dissolved solids and turbidity. A weir makes this area unaccessable to fish, but other freshwater organisms, such as aquatic insects, are present.

The tailing dam lies across the North end of the Tailings Pond. This impoundment is approximately one square mile, and envelopes an area that was once part of the South Fork of Red Dog Creek. The mine facility lies to the northeast of the dam, and includes the mill, water treatment plants, pumphouses, and personnel living quarters. The ore mill lies within the catchment of the tailings pond, allowing seepage to be recovered into the pond. The ore body and the main pit are located on the Middle Fork. A diversion and drainage collection system was built to prevent ore zone runoff from entering the creek. This system also intercepts natural seepage that had been entering the creek prior to the construction of the mine. Effluent discharge is located farther down the Middle Fork, just north of the tailings dam. In 1997, groundwater monitoring and fish protection programs were implemented to study the effects of effluent on local aquatic life.

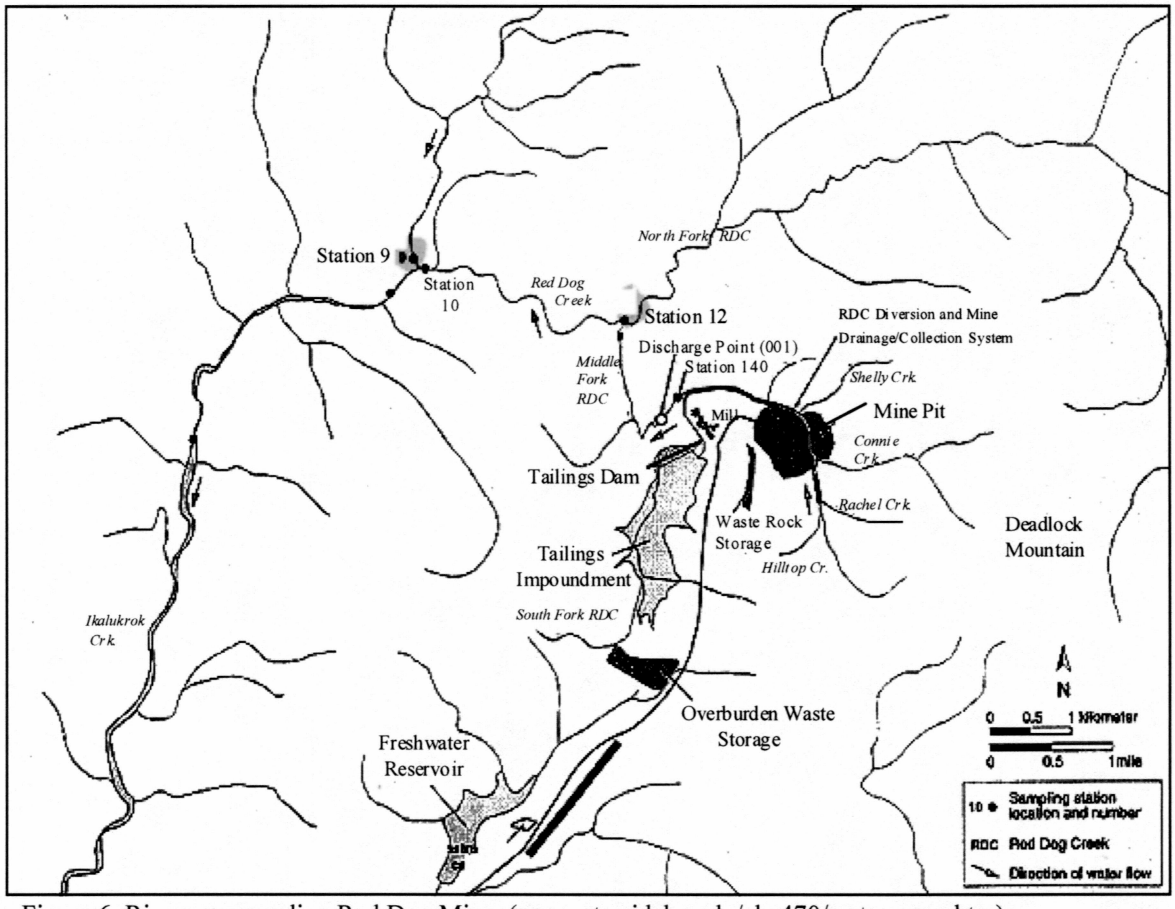


Figure 6. Rivers surrounding Red Dog Mine. (www.ets.uidaho.edu/che470/water_map.htm)

Chapter 2

Bioassays

The *Daphnia magna* test is commonly used as a criterion for the assessment of chronic toxicity in aquatic systems. *Daphnia magna* is among the most sensitive species to a wide variety of environmental contaminates (Hall et al., 1986). The toxicity of many compounds, elements, and mixtures have been measured under a great variety of conditions using *D. magna* (Johnson and Finley, 1980; Cowgill, 1987), and specific sensitivities between *D. magna* and other species are known (Gersich, 1984; Kangarot and Ray, 1987). However, this assay is prone to several complications, especially with life-cycle chronic toxicity studies, due to control mortality and culturing difficulties (Mount and Norberg, 1984).

As alternatives for rapid and relatively inexpensive aquatic toxicity tests, environmental scientists often use freshwater algae, bacteria, and vascular plants to monitor the impact of pollution on freshwater systems. All are primary producers of organic matter, they are essential for providing nutrients to higher trophic levels, and they maintain the balance of dissolved oxygen, nitrogen, phosphorous, and trace elements in the water table (Andreae, 1978). These organisms also contribute to the self-purification of streams, lakes and estuaries. Toxicity assays with bacteria and algae are often used for testing natural waters and effluents because they are less expensive, require smaller sample sizes, have simpler methods, and are quicker than assays using invertebrates or fish (Van Coillie et al., 1983).

Because of the widespread effects of bacteria and algae on the freshwater system, they are excellent indicators of early environmental degradation. Monitoring changes in the behavior of these species can prevent more extensive damage to a habitat at higher trophic levels. Recently, there has been concern regarding the detrimental effects that increasing dissolved solids may have on the growth and development of freshwater organisms. Toxicity tests have been performed on algae and bacterial assays in an attempt to more accurately predict the effects of TDS on higher species in a natural environment.

Most researchers agree that there is no single bioassay that can adequately predict the effects of a toxicant on an entire system (Giesy and Hoke, 1989). Because of the different physiology and biochemistry among organisms, not all toxicants will elicit the same response in all species. In an ideal study, a battery of assays should be performed, which include higher organisms such as fish and invertebrates. Previous studies have indicated that bacteria, in general, have a low sensitivity to metals and no sensitivity to herbicides, while algae is significantly affected by both (Babich and Stotzky, 1985; Giesy and Hoke, 1989). On the other hand, bacteria are much more sensitive to other organic compounds (DeZwart and Slooff, 1983). When compared to *D. magna*, the bacteria *Vibrio fischeri* demonstrated a lower sensitivity to both cadmium and mercury, but a higher sensitivity to copper (DeZwart and Slooff, 1983; Gillespie and Vaccaro, 1978).

One of the advantages of using the bacteria and algae assays is the sensitivity of their endpoints. In being able to closely monitor growth and metabolism, specific effects

on cell development can be used as an endpoint instead of less sensitive events, such as death (Giesy and Hoke, 1989).

2.1 Algae Assays

Algal assays are regularly used for testing the effects of aquatic pollutants on primary producers, and organizations such as the Environmental Protection Agency and the American Society for Testing and Materials have standardized methods for algal assays. Algae is a particularly good indicator of aquatic toxicity because cells are subject to fewer variations, due to fewer functional or genetic diversities in the organisms, compared to bacteria, invertebrates, or fish. In addition, chronic tests can be completed in a relatively short period of time, due to rapid generation time.

Chemical sensitivity tends to vary with algal species. Despite this, a number of studies indicate that they are more sensitive to wastewater samples then other microbial species (Joubert, 1981; Van Coillie et al., 1981), more sensitive to toxins that affect plant cells than bacterial organisms (Beckmann et al. 1984), and more sensitive than other aquatic toxicological short-term screening tests (Walsh et al., 1982; 1984; Adema et al., 1983). However, research has also been conducted that indicates fish and *Daphnia* are more sensitive indicators of toxic effects (Kenaga, 1979; 1982).

Different agencies use different culturing and testing methods, even when using the same species of algae. These variations may involve inoculated cell density, test duration, media used, pH, light, aeration, and agitation. EC_{50} measurements can differ greatly as a result. An external comparison of results from laboratories following different procedures was done by the Nordic Co-operative Organization in 1982. They

found that these factors caused EC_{50} values to differ by over three orders of magnitude (Laake, 1982). Despite these difficulties, algae assays remain important for ecotoxicological hazard assessment, and are key indicators of aquatic environmental health.

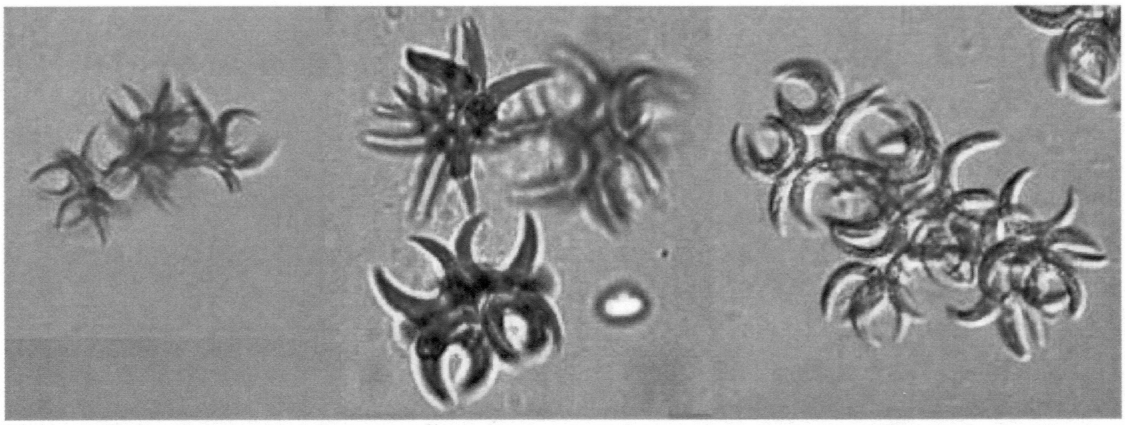


Figure 7. *Selenastrum capriconutum* cells (www.taxa.soken.ac.ip/WWW/PDB/Images/Chlorophyta/Selenastrum/index.htm/).

Selenastrum capricornutum is the standard algal species for determining the toxicity of waters based on algae growth potential, AGP (Giesy and Hoke, 1989). *Selenastrum* is a genius of unicellular, non-motile green algae present in freshwater systems. Cells are sickle shaped, and tend to conglomerate into groups of 10 or more. They are excellent bioassay organisms because they are easy to grow in the laboratory, have short generation times, can be used in small quantities, and are very sensitive to metals and some organic toxicants (Giesy and Hoke, 1989). They are also versatile enough to be used for industrial effluents, as well as natural waters (Joubert, 1983).

Selenastrum capricornutum is a photosynthetic organism containing chlorophylls a and b. The density of the chloropast can easily be measured with a spectrometer, and used to estimate the cell density of a sample. The bioassay procedure is based on the

inhibition of cellular division by the applied toxin. Therefore, the final ratio of absorbance of a test sample to the absorbance of the control can be used to estimate the growth stimulation or toxic effects of the tested water.

2.2 Bacterial Assays

Bacteria are important to the degradation processes of hydrocarbons and nutrients in freshwater systems. Luminescent bacterial assays are used frequently for environmental quality testing. They are versatile enough to be used for monitoring effluent, groundwater, sediment, hazardous wastes, and bioremediation processes (ASTM, 1995). Bioluminescent bacteria use energy derived from respiration to convert chemical energy into visible light. Because this pathway is linked to respiration, any disruption or rate change of this process will be reflected in a decrease of luminescence (ASTM, 1995).

Additional characteristics make luminescent bacteria useful in the laboratory. Most of these species have respiration rates of 10 to 100 times greater than mammalian cells, which produces a highly dynamic metabolic system for testing. These organisms have a short division cycle, and their small size ensures a large surface area to volume ratio, maximizing sample exposure. Most respiratory pathways lie on or near the cell membrane, and cells lack membrane compartmentalization (Bulich et al., 1985). These qualities all allow for a highly sensitive, and easily quantified and reproducible bioassay.

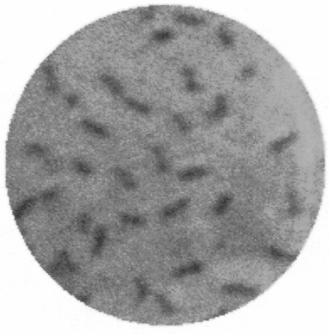
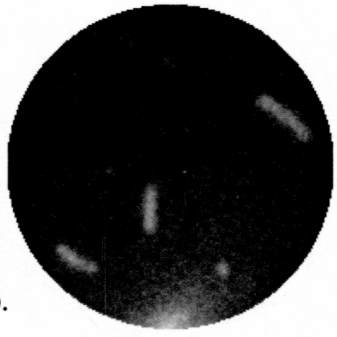
a.  b. 

Figure 8(a) and (b). Microscopic photographs of *V. fischeri* in light (a) and luminescing in dark (b) (P. Madanecki, eniac.farmacja.amg.gda.pl/~microbio/bakterie_sw/bac_zdjm_en.html).

The MicroTox® bacterial assay uses *Vibrio fischeri* (previously *Photobacterium phosphoreum*) to test the toxicity of water samples. *V. fischeri* is a marine bacteria which is usually found naturally in very low concentrations of approximately 100 cells/mL. As a marine organism, the ecological relevance of this species to freshwater systems has been questioned. However, assays with this species of bacteria have been used for over 20 years to test for aquatic toxicity from complex industrial effluents to single chemicals. Large volumes of data, including responses to thousands of chemical compounds, have been collected, and correlations between the responses of *V. fisheri* and freshwater organisms have been well documented (Slooff, 1985). This assay is now accepted by the research community as a first tier screening procedure for aquatic toxicants (Slooff, 1985)

AZUR Environmental has developed a commercial method for the screening of surface water, groundwater, wastewaters, leachates, and organic extracts based on this

assay. Numerous publications have documented the reliability of this procedure, which is now used by government monitoring agencies as a cost effective, reliable, and reproducible test to measure the toxicity of environmental samples. Previous studies have demonstrated an agreement between toxicity determined by the MicroTox®, fathead minnows (*Pimephales promelas*), and *Daphnia magna* (Greene et al. 1985). Slooff has found correlations between the MicroTox® and assays using insects, invertebrates, protozoans, and fish.

The MicroTox® assay uses pre-packaged, freeze dried organisms, disposable test cuvettes, and a specialized luminometer for the assay procedure. The standardization of materials and methods eliminates many of the complicating factors and sources of uncertainty inherent to bioassay experiments. Samples are read on a temperature controlled, bioluminometer with the use of a photomultiplier tube. The instrument used in this study, a MicroTox® model 500 Analyzer, incubates samples at 27° C for optimal growth during the test. The observed growth capacity (luminescence) of the exposed bacteria and the control bacteria is measured and compared after 24 hours. This 24 hour period is long enough to include multiple life cycles of the test organisms (AZUR, 1998). Photoemission from the luciferase-catalyzed reaction is read at 490nm (Briscoe et al., 1998). A method blank is used to calibrate the analyzer prior to the sample readings, and a 10ppm Cu^{++} ($CuSO_4$) standard is used as a positive control for each batch of samples.

Although the MicroTox® instrument is capable of both chronic and acute toxicity testing, our study focused on the chronic assay analysis (These laboratory tests require separate test medium, supporting solutions, and laboratory equipment.). The intent of the

chronic test is to determine the effects of toxicant exposure over an organism's entire life cycle, which reflects the results of constant exposure of aquatic life to dissolved solids in the water column.

Table 3. Method comparison between assays

Assay	Species	Replicates	QC	Blank	Time
Algae	*Selenastrum capricornutum*	3	none	yes	72 hours
MicroTox®	*Vibrio fisheri*	2	$CuSO_4$	yes	24 hours

Neither the MicroTox® assay nor the algae assay have previously been used to test for the effects of TDS in produced waters.

Chapter 3

Methods

3.1 *Sampling*

Water samples from Fort Knox Mine were taken every four weeks from June to November, 1999. Sampling stations at Fort Knox Mine included the Tailings Pond decant, Interceptor Well #3, Monitoring Well #3 , and the Freshwater Reservoir. Water was collected from the pump port at each well site, and as grab samples from the Tailings Pond and the Freshwater Reservoir. Samples were collected into polystyrene containers with minimal headspace. All samples were kept at 4° C during transportation and storage. Temperature and pH readings were made on site, at the time of sampling.

Red Dog Mine samples were taken monthly from four sites, and sent to the University of Alaska by Cominco staff. These sites included the location of the outfall (001); on the Middle Fork of Red Dog Creek, just down river from the mine (Station 140); on Red Dog Creek, far down river form the mine (Station 10); and at the mouth of V. River, a tributary to Red Dog Creek (Station 9). Upon arrival at the laboratory, they were stored at 4° C until analysis. The pH was measured and recorded prior to analysis.

Not all samples were shipped cold to UAF. Warming to room temperature may have affected the pH of the water samples. As previously mentioned, the toxicity of some components in water samples, such as metal ions, can be pH sensitive. Preliminary studies conducted at the State of Alaska Department of Fish and Game have determined that the changes in toxicity of effluent water samples, at these temperatures, are negligable (Weber, 2000).

3.2 *Synthetic TDS Solution Materials*

The synthetic TDS solution was made in water treated by reverse osmosis. All salts were reagent grade, purchased from Aldrich Chemical (Milwalkee, Wisconson). Solutions were stored at 4° C for up to three months.

3.3 *Algal Assay*

3.3.1 *Materials*

Selenastrum capricornutum cultures were purchased from UTEX (Austin, Texas). Media, and polystyrene cells were purchased from Creasel (Brussels, Belgium). A Spectronic Genesys 2 spectrophotometer (Rochester, New York) was used for absorbance measurements.

3.3.2 *Preparation and Culture of Selenastrum capricornutum*

To prevent bacterial contamination, all media and glassware were autoclaved prior to use, and all transfers made under an evacuation hood.

Working assay solutions were made by transferring cells from the culture to 250mL flasks containing approximately 100mL media. These flasks were placed on a shaker and exposed to full spectrum light for approximately 14 days. Once absorbance of the stock culture reached at least 1.10 at 670nm, it was used for sample testing. This corresponds to a cell count of greater than 1.1 million cells per mL.

Calibration of cell density to spectrometric absorbance was determined by microscopic cell count using a hemacytometer. This calibration was conducted once at the beginning of the study, and repeated at the end of the sample testing.

3.3.3 *Algal Assay Procedure*

Five 200mL volumetric flasks labeled 1 through 5 were used for serial dilutions. An additional 200mL flask, labeled 0 served as the control. A 100mL aliquot of medium was added to flasks 0, and 2 through 5. A 200mL aliquot of effluent was added to flask 1. Serial dilutions of 2:1v/v were made by transferring 100mL from flask 1 to flask 2, 100mL of flask 2 to flask 3, and so forth. The final 100mL of flask 5 was discarded. Ten mL of the algae stock solution was added to each flask. The final sample concentrations of the labeled flasks are listed in Table 4.

Table 4. Dilution series for use with the Algal Assay

Flask number	Sample Concentration (%)
0	control
1	100
2	50
3	25
4	12.5
5	6.25

From these solutions, 25mL samples were poured up in triplicate using 10cm polystyrene cells. The absorbance of each was measured at 670nm. This measurement is directly related to the algae cell density of each sample. Samples were incubated at room temperature, 20° to 25° C, using full spectrum light, and measured every 24 hours for a total of 72 hours. The cell density values were used to calculate cell growth, by using the following formulae.

$A =$

$$[(N_1 - N_0)\, t_1]\,(1/2) + [(N_1 + N_2 - 2N_0)(t_2 - t_1)]\,(1/2) \cdots + [(N_{n-1} + N_n - 2N_0)(1/2)](t_n - t_{n-1})$$

where: A = area under double linear growth curve for each sample,
t_1 = time of first measurement after beginning of test,
t_n = time of nth measurement after the beginning of the test,
N_0 = nominal initial cell density,
N_1 = measured cell density at time t_1, and
N_n = measured cell density at time t_n;

$I_{Ai} = (A_c - A_i)(1/A_c) \times 100$

where: I_{Ai} = percentage inhibition for test concentration i,
A_i = mean area for test concentration i, and
A_c = mean area concentration for control.

3.4 *MicroTox® assay*

3.4.1 *Materials*

The MicroTox® Chronic Test Reagent (2% *V. fisheri,* 2% NaCl, 96% carbohydrates and pH stabilizers), Reagent Activation Solution (3.5% NaCl solution), Test Medium (94.36% mixed salts, 0.46% magnesium sulfate, 0.01% peptone, and 1.42% potassium chloride), Reconstitution Solution (0.01% sodium chloride), cuvettes, support blocks, and MicroTox® model 500 Analyzer were purchased from AZUR Environmental (Carlsbad, California). $CuSO_4$ was purchased from Aldrich Chemical (Milwaukee, Wisconsin).

3.4.2 *Procedure*

The MicroTox® Chronic Toxicity Test REAGENT is stored freeze-dried in 36mL aliquots, each containing approximately 50 million organisms. The Chronic Media is

also stored freeze dried. These reagents must be kept between -20° and -25° C until analysis.

Cuvettes were loaded into rows A through I of the support blockwith an additional cuvette in the labeled reagent well of the instrument. This well is temperature controlled at 15°C. Three mL of the reagent activation solution were added to the REAGENT well cuvette and allowed to equilibrate in the instrument for five minutes. This solution was used to rehydrate one vial of REAGENT by quickly pouring it into a reagent vial, swirling, and transferring it back to the cuvette. The cuvette is returned to the REAGENT well and the solution thoroughly mixed by filling and dispensing a one mL pipettor three times.

One vial of test medium was rehydrated using 36mL of room temperature reconstitution solution. Again, the resulting solution was thoroughly mixed with a pipette. A 250μL aliquot of reagent from the REAGENT well cuvette was added to the rehydrated medium and thoroughly mixed. A 500μL aliquot of the rehydrated reagent/medium solution was added to each cuvette of columns one through four of the support block. One mL of this same solution was added to each cuvette in column five of all rows.

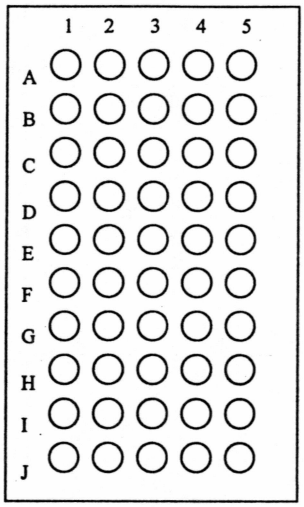


Figure 9. MicroTox® luminometer sample well grid.

A 36mL aliquot of room temperature sample was added to a new vial of test medium and thoroughly mixed. Again, 250μL of rehydrated reagent was added to the sample/medium solution. One mL of the sample/reagent/medium was added to cuvettes C5 and D5 and the unused portion of the sample/reagent/test medium discarded. This process was repeated for additional samples.

Ten μL of the 10ppm Cu^{++} solution, from the working solution $CuSO_4$ were added to cuvette B5. This sample served as a quality control (QC).

Serial dilutions of 2:1 v/v were made using the blank, QC, and samples, in which 500mL aliquots were transferred from one cuvette to the next in the following order:

A5 to A4
A4 to A3
A3 to A2
A2 to A1

The final 500µL from A1 was discarded and each cuvette thoroughly mixed after each transfer.

This pattern of dilutions was repeated for all rows, with the final 500µL from the last cuvette in each row discarded. All cuvettes were incubated uncovered at room temperature for 22 hours.

After this time, the cuvettes were read by the luminometer. The instrument was calibrated prior to sample readings by placing the cuvette A1 in the "READ" well and is depressing the "SET" button. If light readings read between 90 units, sample incubation was continued for 24, or if necessary, 26 hours. At this point, the test was aborted if the controls did not read above 90 light units. All samples were read as prompted by the program. Effects were calculated using the following formulae:

$\Gamma = (I_c - I_s) - 1$ where: I_c = average light intensity of control samples,
I_s = light intensity of sample; and

% effect = $[\Gamma / (1 + \Gamma)] \times 100$.

3.5 *Laboratory Analysis*

Northern Testing Laboratories in Fairbanks and CT&E Laboratories in Anchorage analyzed all samples for Total Dissolved Solids (TDS), chloride, sulfate, sodium, calcium, potassium, and magnesium. TDS was measured according to Standard Method 2540C. This method involves filtering the sample through a micropore filter into a crucible, dried at 180° C for at least 2 hours, and weighed.

Anions chloride and sulfate were measured on an ion chromotographer by EPA Method 300. Cations sodium, calcium, potassium, and magnesium were measured on an inductively-coupled atomic plasma spectrometer (ICP) by EPA Method 6010.

All other analyte concentrations were measured by independent laboratories under contract to Fort Knox and Red Dog mines, in compliance with EPA for monitoring purposes.

Chapter 4

Results

4.1 *Effects of storage*

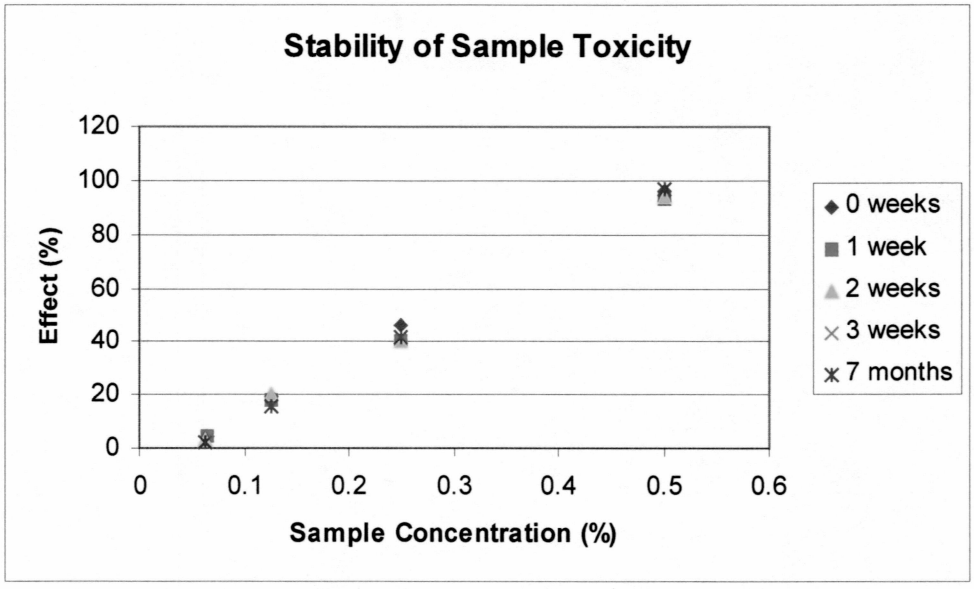


Figure 10. Results of Fort Knox tailings pond sample (6/24/99), tested on the MicroTox® assay after periods of storage.

The effects of storage time were studied by measuring a tailings sample from Fort Knox Mine over a period of seven months. The results demonstrated that toxicity is constant over this period of time, when the samples are stored at 4° C. Occasional warming of the samples to ambient temperature did not appear to have a significant effect on the toxicity of the samples.

4.2 *Reporting of Results*

In this study, EC_{20} values were chosen as the reported toxicity value for the samples. This is largely due to the fact that most samples were not toxic enough to

produce EC_{50} values. Although many studies present results as EC_{50} values, the EC_{20} result is a more sensitive indicator of environmental toxicity.

Toxicity results were calculated using EC_{20} values in percent sample concentration. To compare results between different samples, these percent concentrations were multiplied by the total TDS concentration for that sample. The resulting toxicity values of EC_{20} are in mg/L TDS.

Reporting toxicity values of these samples relative to TDS concentrations was intended to illustrate whether or not the EC_{20}'s corresponded to a consistant level of TDS among the samples. If the results demonstrated consistancy, it would indicate that the toxic responses from the samples could be attributed to TDS levels. Inconsistancies between the synthetic and field sample EC_{20}'s would likewise indicate that the observed toxic results are likely due to some other component.

4.3 *Sample Composition*

The chemical composition of the field samples are given in appendix A.

4.4 *Comparison of Results Between Assays*

Table 5. Assay Results*

Sample	Date	MicroTox EC_{20} (mg/L TDS)	Algal EC_{20} (mg/L TDS)	Total TDS (mg/L)
FK IW#3	6/24/99	334	NT	380
FK Tailings	6/24/99	1.15	80.0	870
FK IW #3	7/22/99	NT	44.8	358
FK Tailings	7/22/99	0.41	NT	730
FK Tailings	8/26/99	1.40	123	650
FK Tailings	9/23/99	1.29	31.9	558
FK Tailings	10/25/99	0.41	NT	578
RD St. 140	6/8/99	NT	NT	94
RD St. 140	7/13/99	111	42.7	356
RD St. 140	8/10/99	239	NT	251
RD St. 140	9/14/99	NT	NT	323
RD St. 140	10/5/99	NT	215	361

*Only samples producing EC_{20} values on at least one assays are included. FK = Fort Knox Mine, RD = Red Dog Mine, NT = non-toxic, samples did not produce a toxic response to 20% or more of the assay organisms.

Of the samples tested, few demonstrated toxic effects while most stimulated growth of both assay organisms. Due to the limits of the assays, controls, and instruments, stimulatory effect were difficult to compare between runs and samples. For this reason, all readings demonstrating stimulatory effects were classified qualitatively as Non-Toxic, NT. Initially, it was observed that most samples giving stimulatory responses on the MicroTox® gave similar results on the algae assay. To save time and resources, the MicroTox® assay was used to screen out non-toxic samples. Only those which gave positive (toxic) results were tested on the algal assay. It was observed, however, that water samples from Red Dog Mine Station 140 and Fort Knox IW#3 sometimes produced greater toxic effects on the algal assay than the MicroTox®. These samples were therefore tested on both assays regardless of the MicroTox® result.

4.5 *Red Dog Mine Samples*

Most samples from Red Dog Mine also exhibited non-toxic effects on the MicroTox® and algal assays, including all those taken from Station 001, Station 9, and Station 12. Several samples from Station 140 inhibited cell growth, July being the most toxic with an EC_{20} of 111 mg/L TDS. Samples taken in June, September, and October tested non-toxic. The July sample from St. 140 also tested most toxic on the algal assay, with an EC_{20} of 42.7mg/L TDS. Samples taken in June, August, and September tested non-toxic to *S. capricornutum*. This was the only sample site for which the algal assay was consistently more sensitive than the MicroTox®.

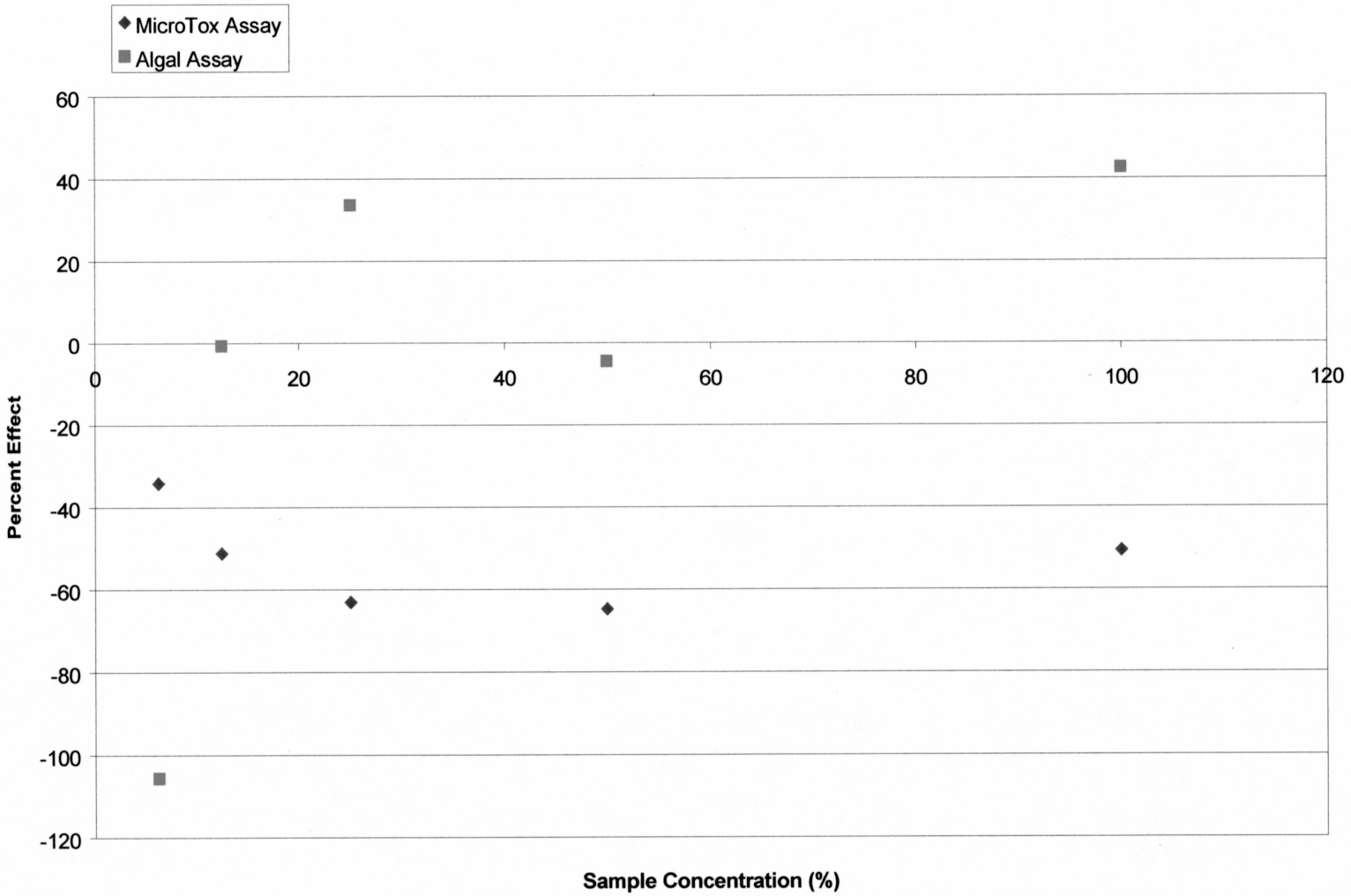


Figure 11a. Dose-response curve for Red Dog Mine, Station 140, June, 94mg/L TDS.

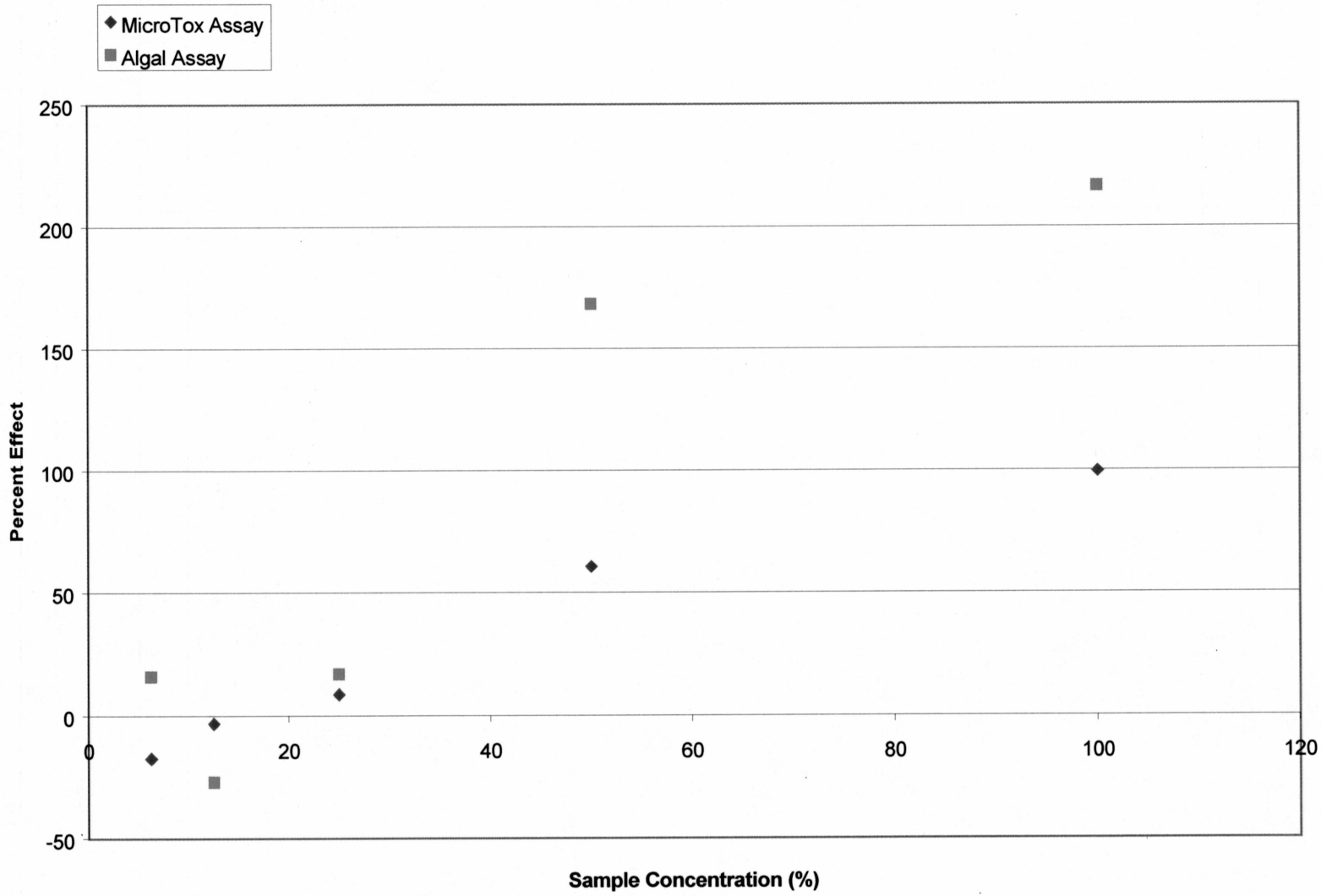


Figure 11b. Dose-response curve for Red Dog Mine, Station 140, July, 356mg/L TDS.

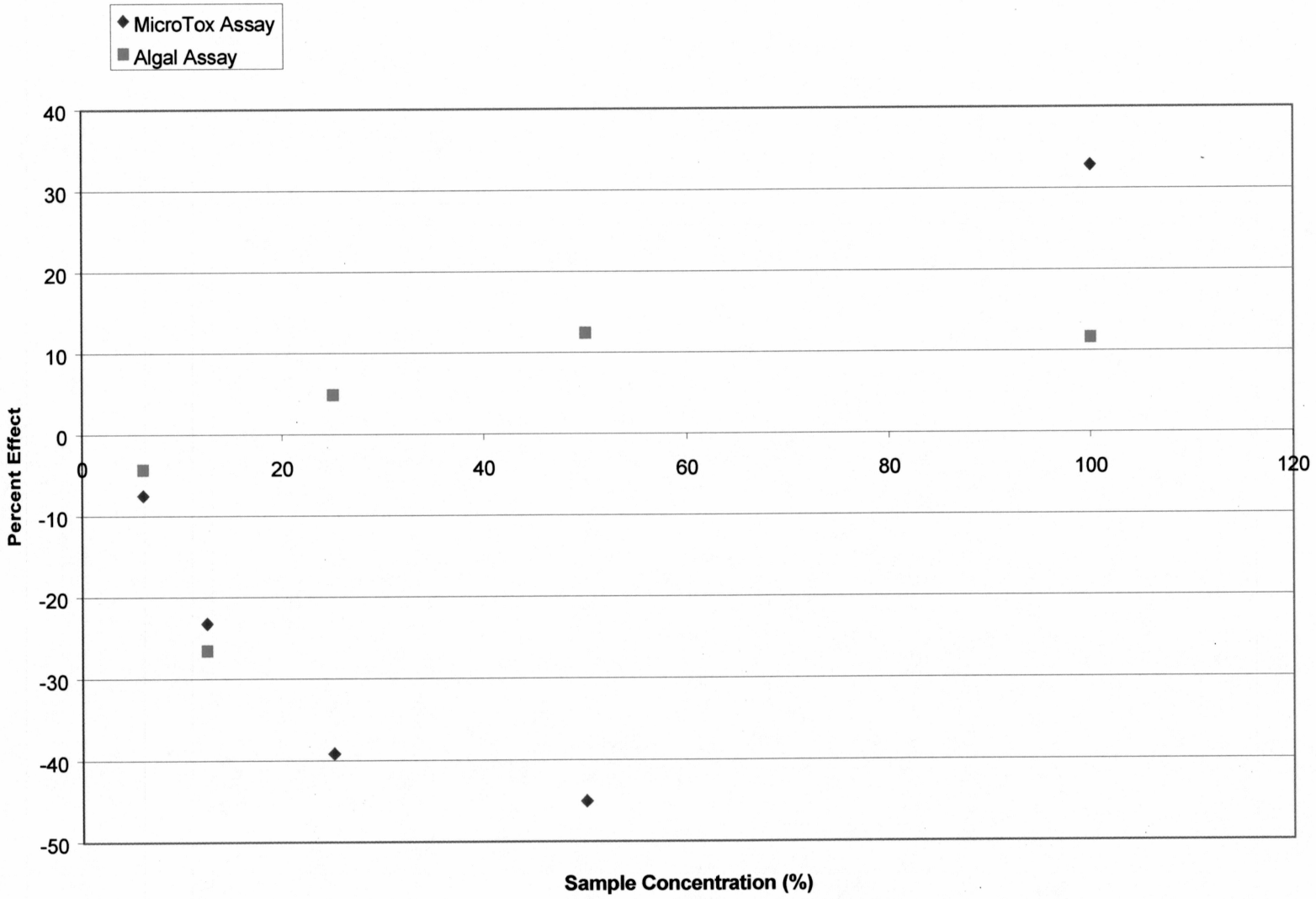


Figure 11c. Dose-response curve for Red Dog Mine, Station 140, August, 251mg/L TDS.

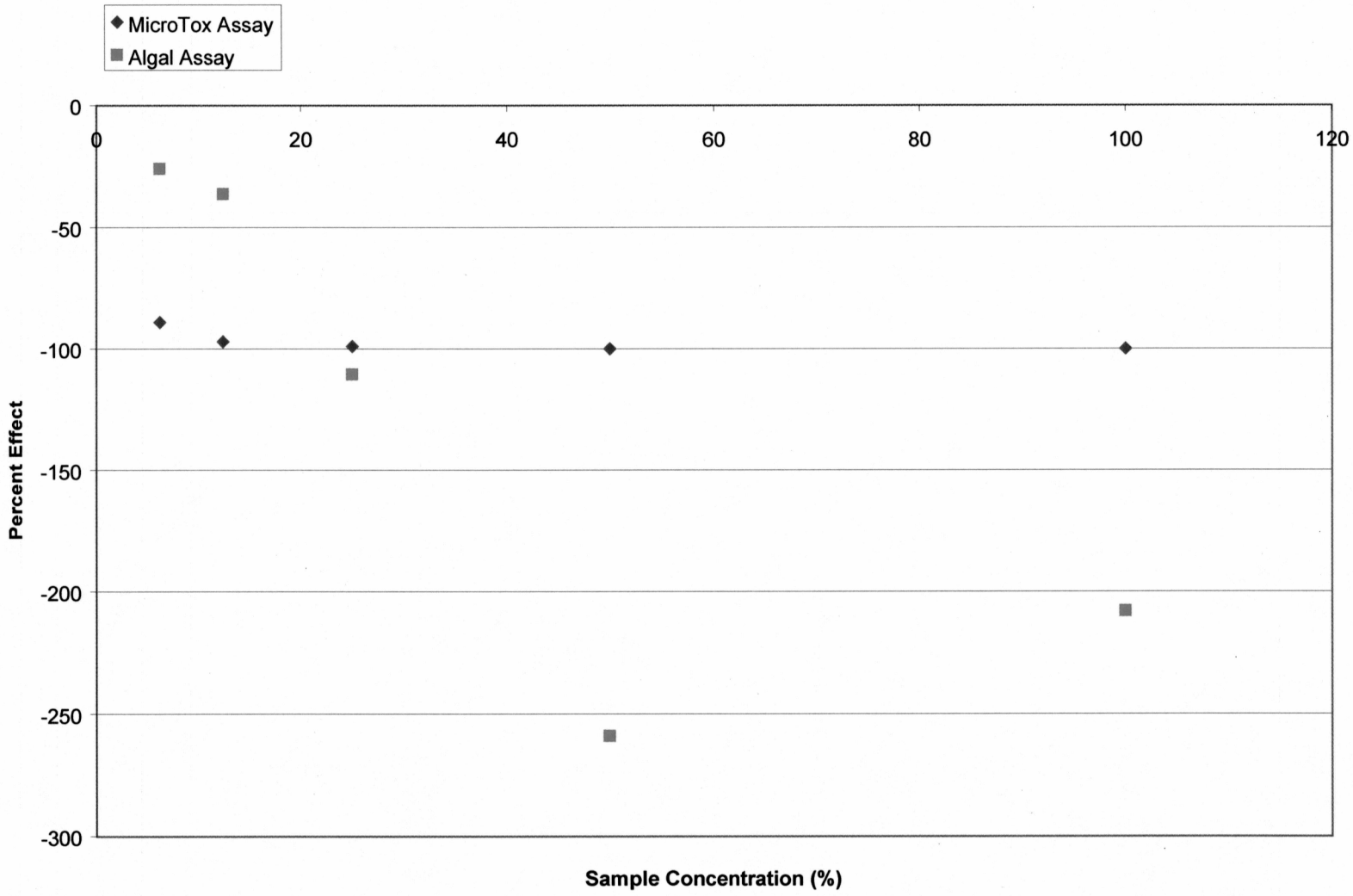


Figure 11d. Dose-response curve for Red Dog Mine, Station 140, September, 323mg/L TDS.

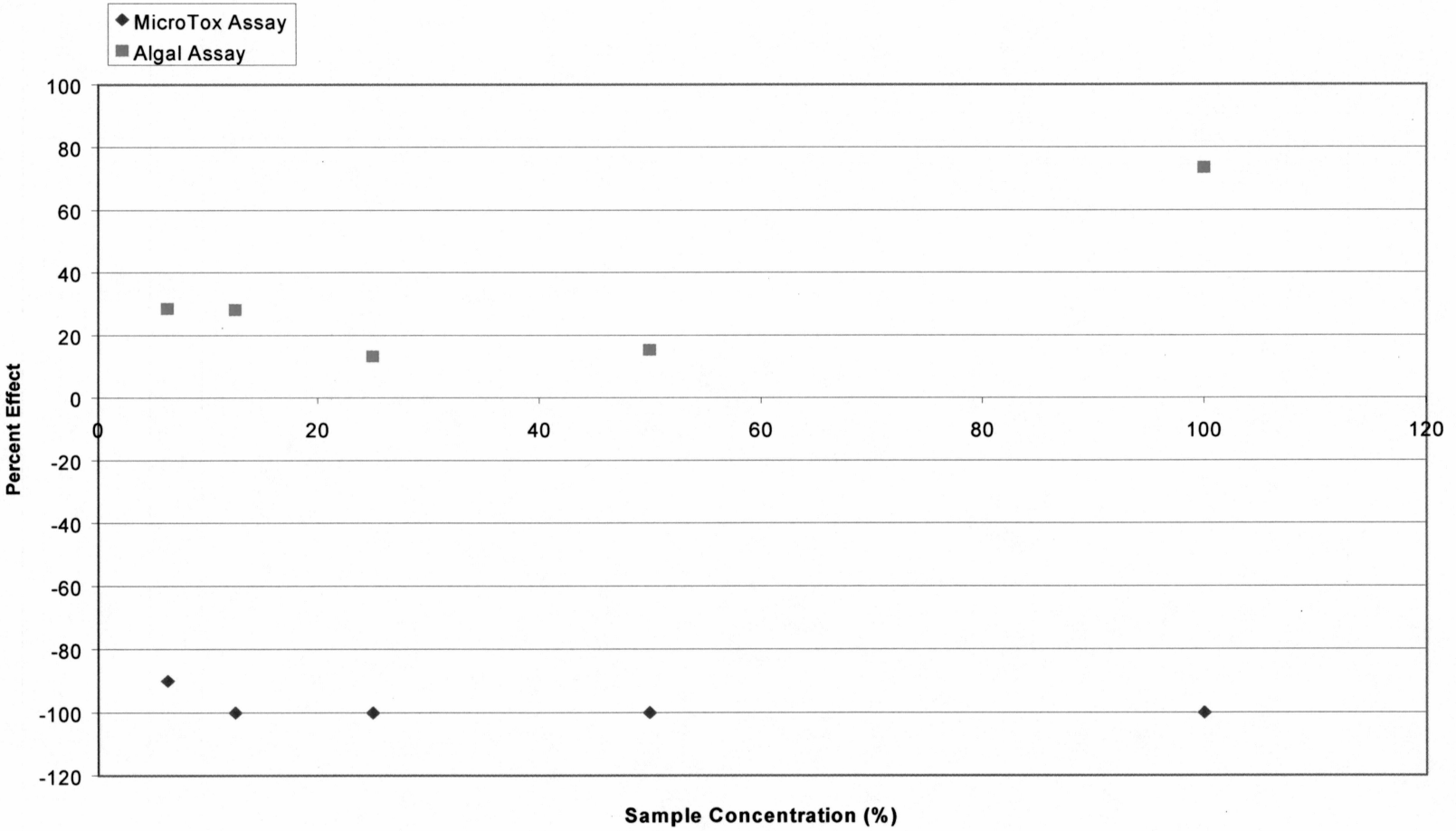


Figure 11e. Dose-response curve for Red Dog Mine, Station 140, October, 361mg/L TDS.

4.5 *Fort Knox Mine Samples*

Few of the Fort Knox Mine samples tested on the MicroTox® showed growth inhibition of *V. fischeri*. Only the Tailings Pond samples continually demonstrated toxic effects during the sampling period. EC_{20} values derived from the MicroTox® assay were fairly consistent, varying between 0.41mg/L TDS in July to 1.4mg/L TDS in August. Samples from IW #3 produced toxic effects only in June, and had an EC_{20} of 334 mg/L TDS. The Freshwater Reservoir and MW #3 consistently exhibited non-toxic effects.

The algal assay, with one exception, proved to be much less sensitive than the MicroTox® assay when testing Fort Knox Mine samples. EC_{20} values for the Tailings Pond samples varied between 32mg/L TDS in September, to 123mg/L TDS in August. The July sample, which demonstrated the most toxic effect on the MicroTox®, demonstrated *no* toxic effects on the algal assay.

The one exception was the July IW#3 sample, which tested non-toxic on the MicroTox®, but generated an EC_{20} of 44.75 mg/L on the algal assay

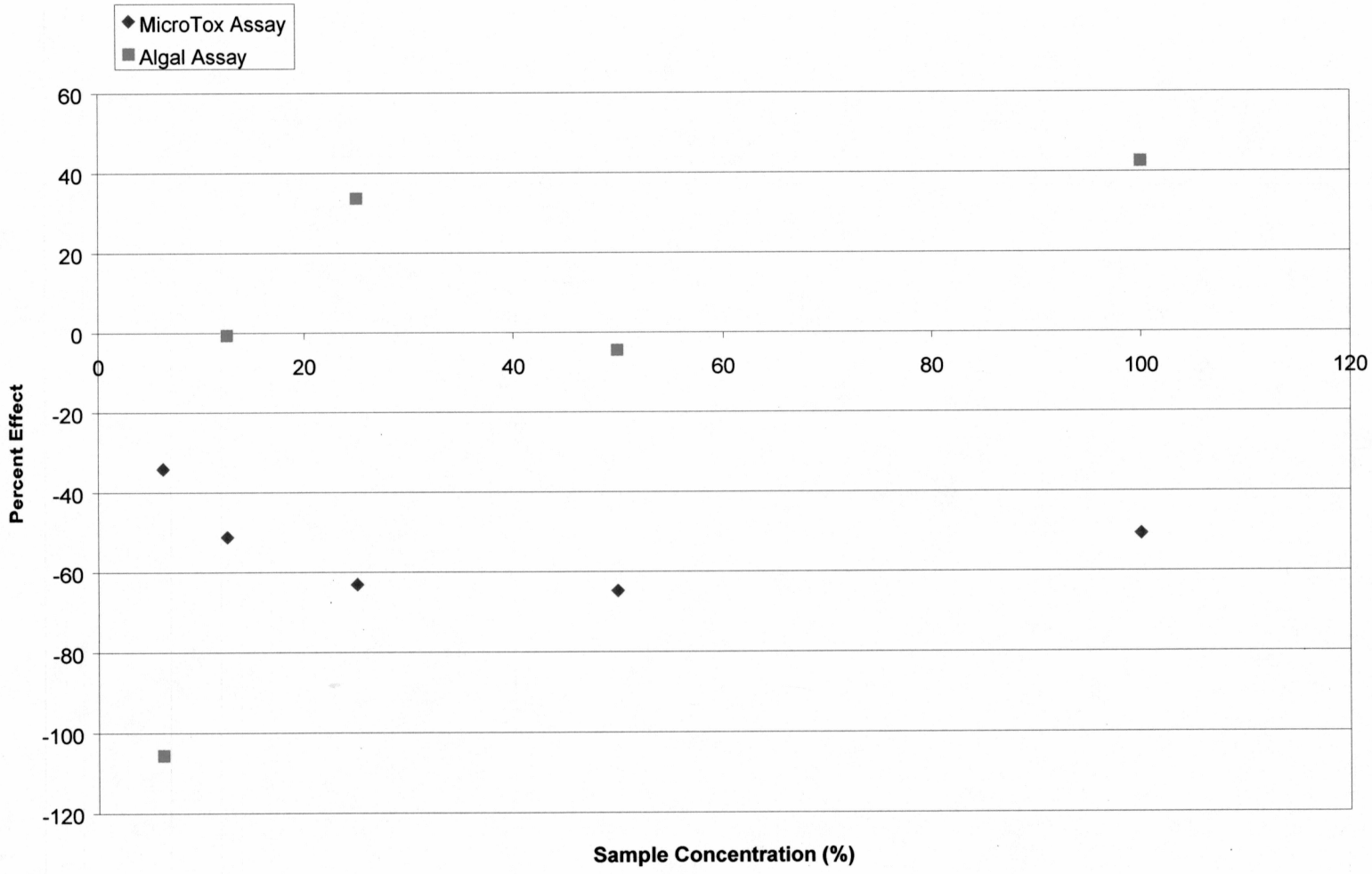


Figure 12a. Dose-response curve for Fort Knox Mine, Tailings Pond, June, 870 mg/L TDS

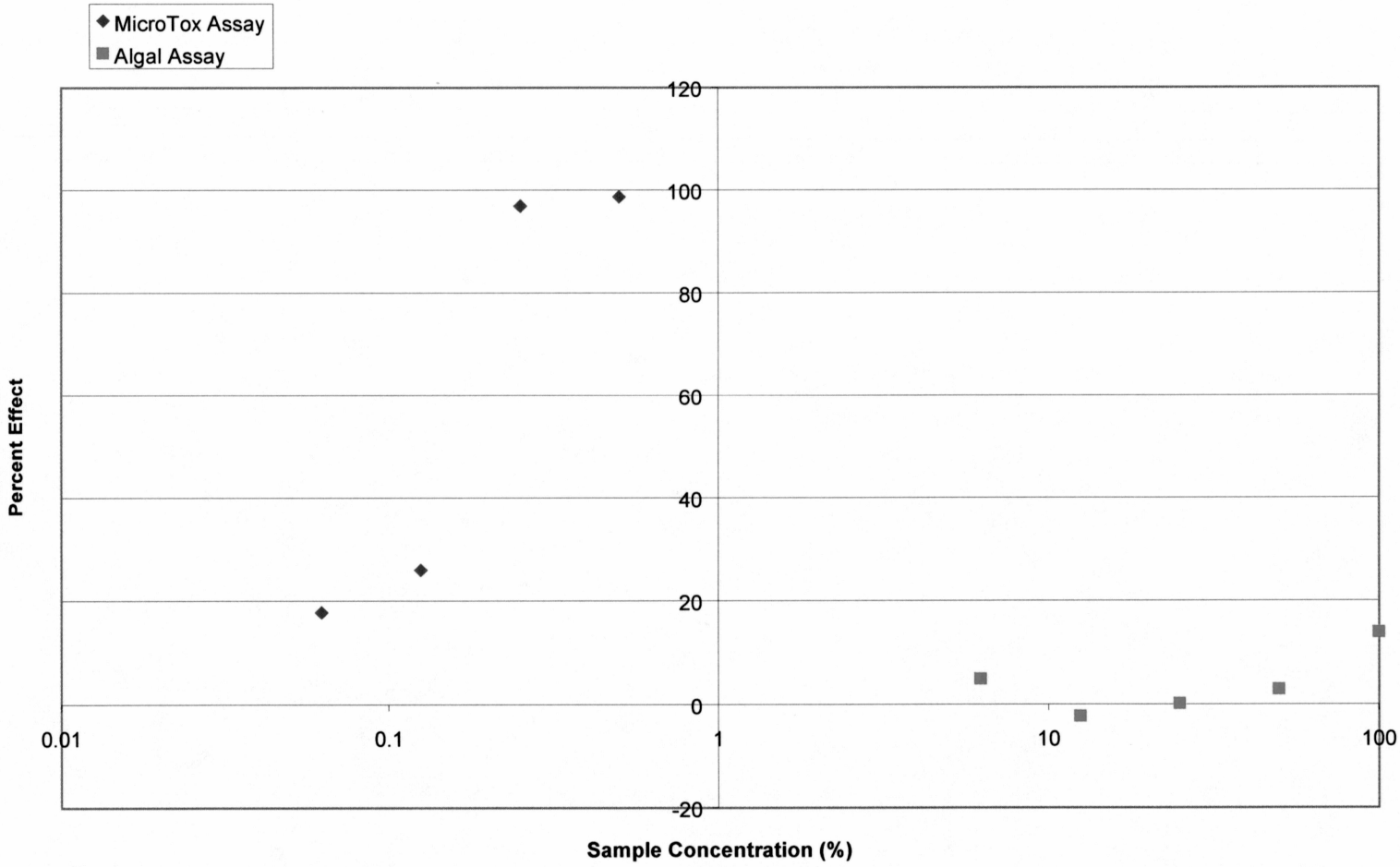


Figure 12b. Dose-response curve for Fort Knox Mine, Tailings Pond, July, 730 mg/L TDS

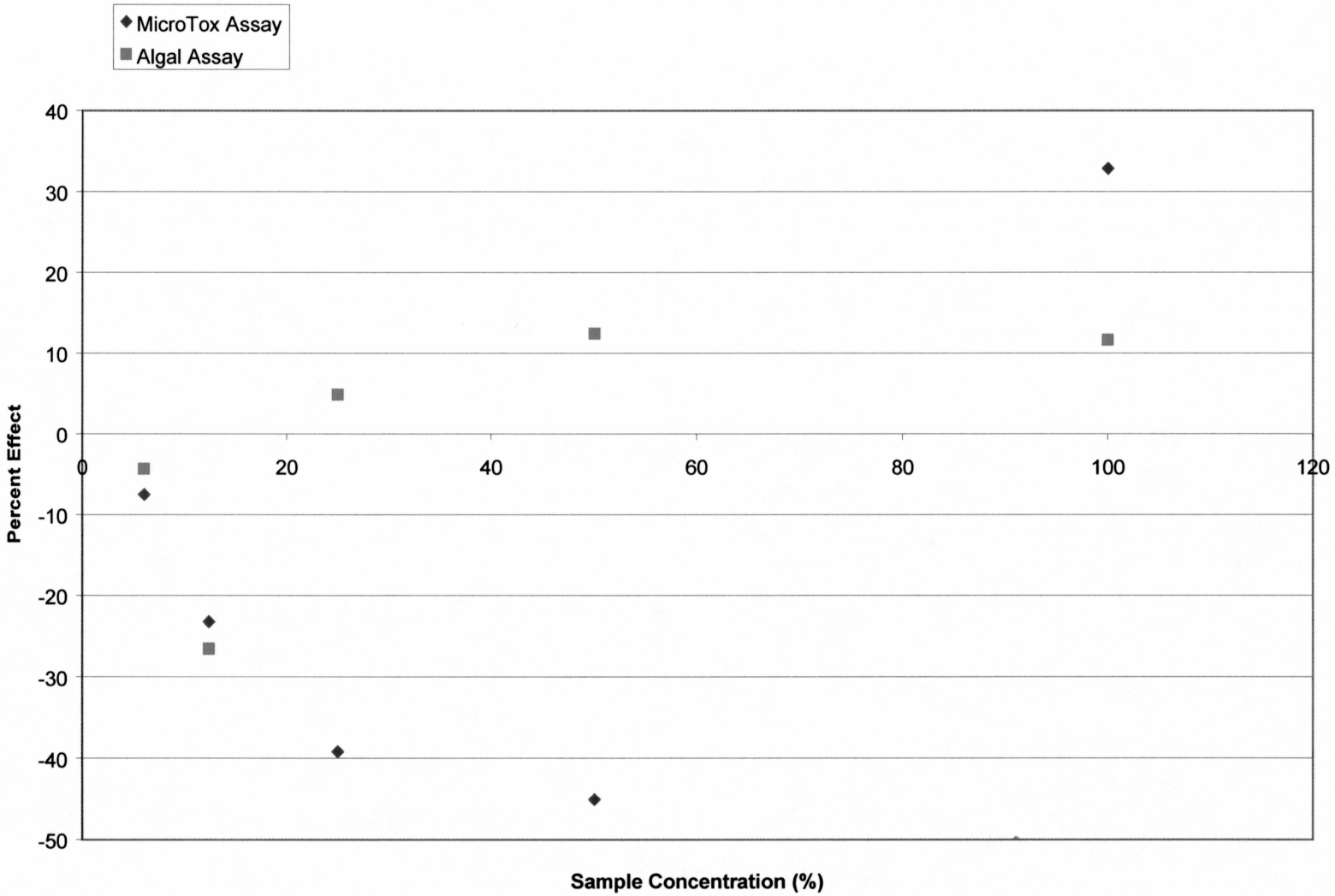


Figure 12c. Dose-response curve for Fort Knox Mine, Tailings Pond, August, 650mg/L TDS

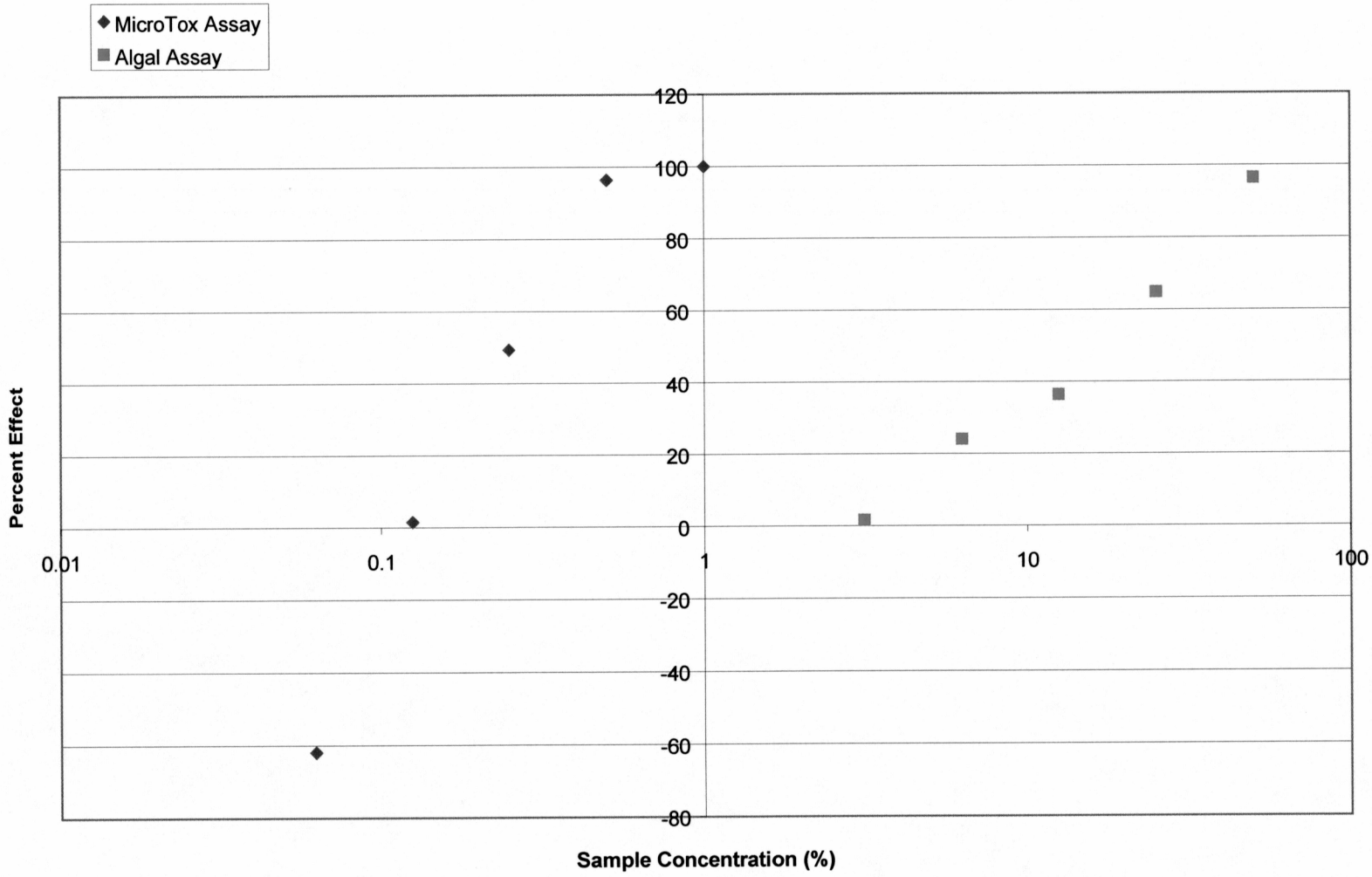


Figure 12d. Dose-response curve for Fort Knox Mine, Tailings Pond, September, 558mg/L TDS

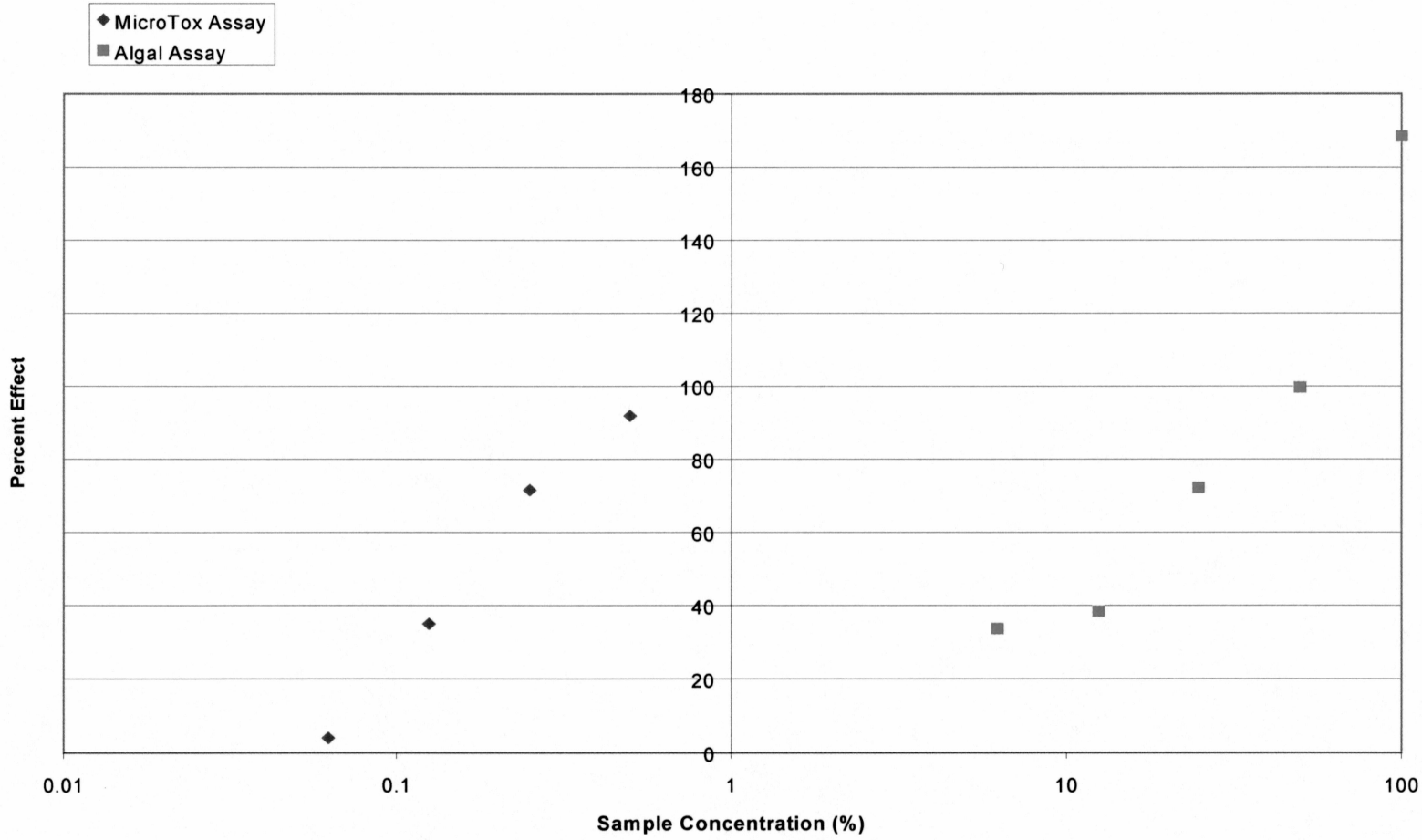


Figure 12e. Dose-response curve for Fort Knox Mine, Tailings Pond, October, 578mg/L TDS

4.7 *Synthetic TDS solution*

The 2500 mg/L synthetic TDS solution made in the laboratory demonstrated significantly different results than the field samples on both the MicroTox® and algal assays. When run on the MicroTox® instrument, samples between 156 mg/L TDS and 1775 mg/L TDS demonstrated non-toxic effects. Samples concentrations between 1775 mg/L TDS to 2500 mg/L TDS led to inhibited cell growth, and had a measured EC_{20} value of 1960mg/L TDS. On the algal assay, however, all sample concentrations demonstrated toxic effects, with an EC_{20} value of 551.3mg/L TDS.

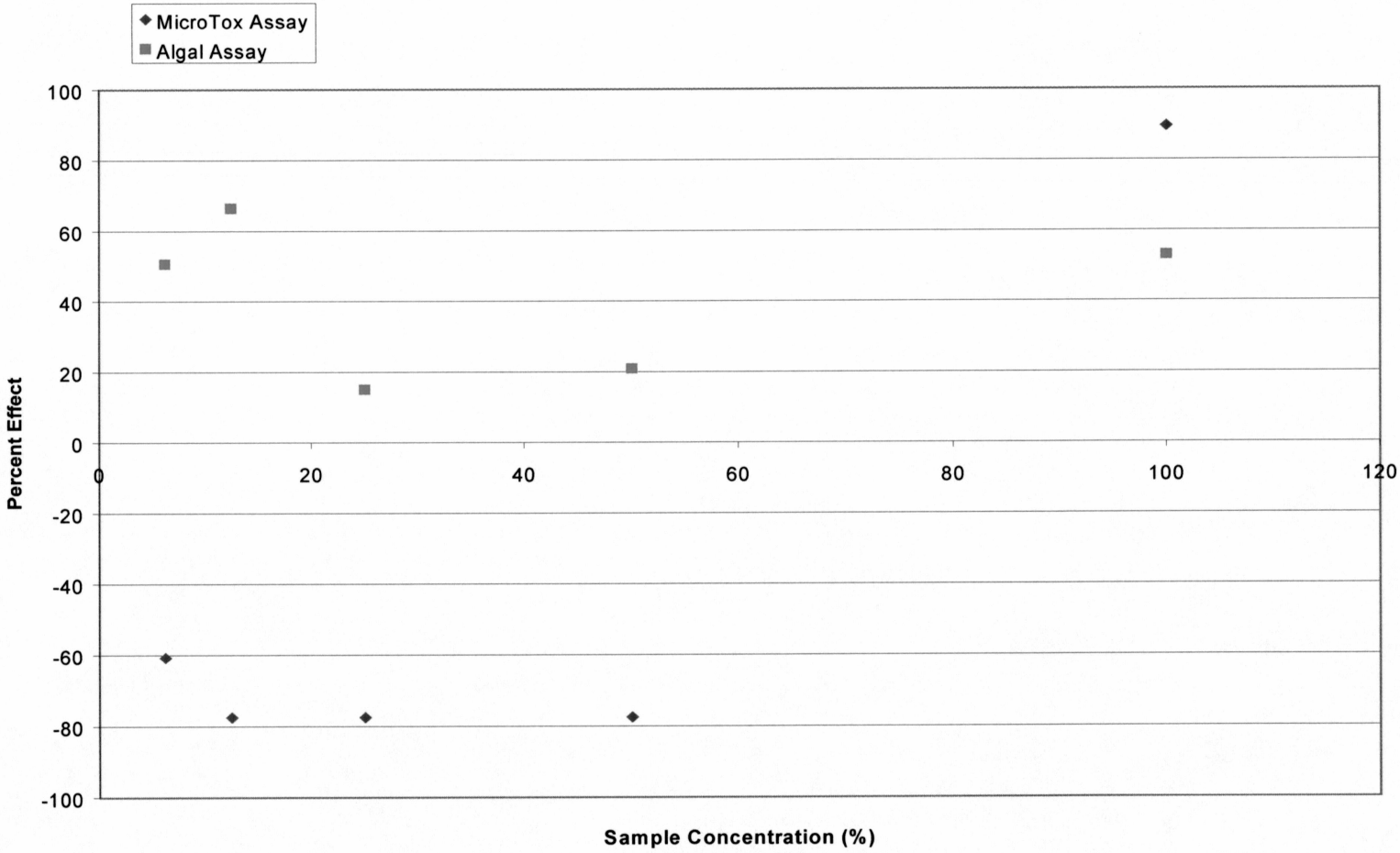


Figure 13. Dose-response curve for synthetic TDS sample.

4.8 *Dilution Effect on Toxicity*

Throughout the assay testing procedures, media was used to dilute samples. To simulate more realistic circumstances, such as effluent being diluted as it is released, a brief set of tests was done using water samples from Red Dog Mine. Effluent was diluted with water having similar ion composition as the receiving waters of Red Dog Creek, and tested at various concentrations. This was done to check for synergistic effects of mixing outfall and receiving water, which may change the toxicity of the mixture beyond that which is observed from the outfall and receiving waters individually.

The receiving waters of Red Dog Creek are composed of approximately 25% Station 140 water, and 75% Station 12 water. "Receiving water" of 3:1 v/v composition was prepared in the laboratory from Station 140 and Station 12 samples, taken on 7/13/99. Assuming no precipitation upon mixing, the resulting TDS of this solution becomes 386mg/L. This water was mixed in various proportions with water from the outfall (Station 001). These mixed samples, as well as the "receiving water" solution were tested using the MicroTox® assay. Results of each solution individually were compared to those of the mixed samples.

There was no observable increase in toxicity of the "receiving water" when mixed with effluent. Both individual and mixed solutions were non-toxic at all concentrations tested.

For comparison, the sample procedure was repeated with the synthetic TDS solution instead of effluent. Solutions were made using the "receiving water" solution as

the dilutent, and run on the MicroTox®. This procedure was repeated using RO water as the dilutent. Results are illustrated in Figure 14.

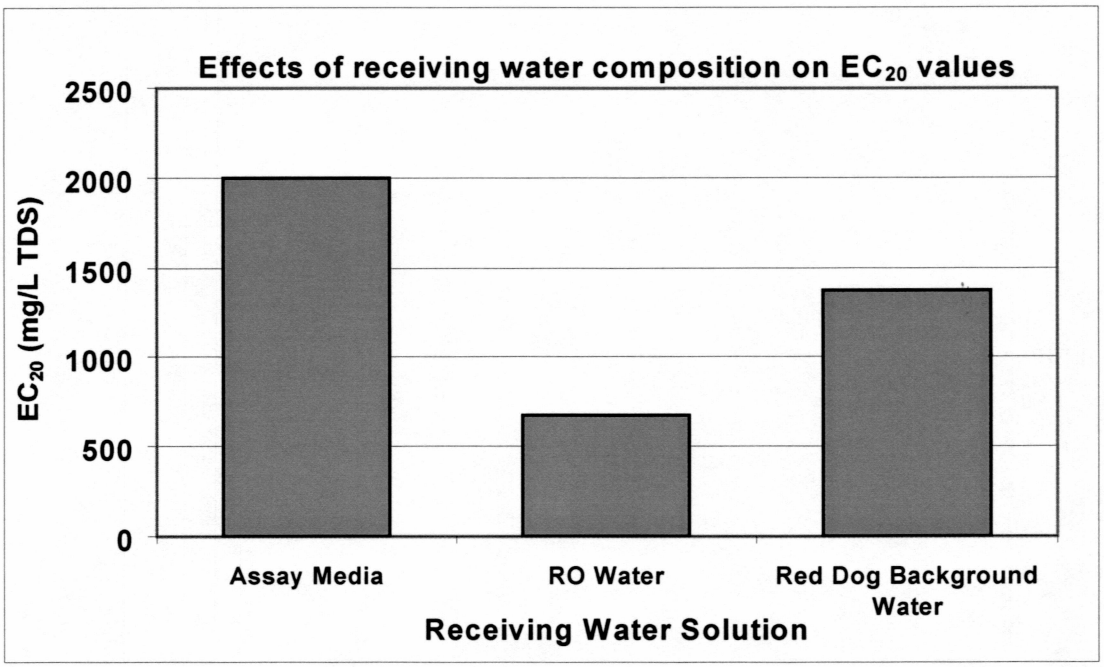


Figure 14. Effects of receiving water composition on EC20 values of the synthetic water solution. (as measured on the MicroTox® assay). Red Dog background water consists of 25% St. 140 water and 75% St. 12 water.

There was an observed increase in toxicity of the synthetic TDS solution when using "receiving water", instead of media as the dilutent. RO water from the lab further increased the toxicity of the synthetic solution to the bacterial cells. These results indicate that the composition of the receiving water can also influence the effects that discharged effluent may have on freshwater biota.

The most likely explanation for the observed effects lies in the importance of dissolved material to aquatic organisms. RO water is totally devoid of these dissolved ions, and is therefor nutrient difficient. The synthetic TDS solution has a moderately high level of dissolved ions, which stimulates growth at low to medium concentrations.

As RO water dilutes the ionic concentration in the synthetic TDS solution, the resulting toxicity of the combined solutions falls between that of each solution individually. The ion concentration of the "receiving water" is between that of the RO and the media, and does not dilute the TDS concentration of the synthetic TDS solution to the degree that RO water does. The result is an EC_{20} that is slightly greater (indicating less toxicity) with the "receiving water" dilutent than with the RO water dilutent.

4.9 *Laboratory Samples*

In addition to running field samples, laboratory standards composed of TDS ions were tested on the assays. *Design Expert* (Minneapolis, Minnesota), a factorial design program was used to design and interpret the assay result of these standards. *Unscrambler,* a multi-varient modeling program was used to construct toxic response models for each of the assays. Field samples and ion standards were modeled separately. The ion standard solutions consisted of mixed and individual ions in the following concentrations (Table 6):

Table 6. Laboratory Standards of TDS Ions

	TDS (mg/L)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)	Cl (mg/L)	SO4 (mg/L)
Ca Std	3500	975	0	0	0	0	2525
Na std	3950	0	0	0	565	0	1177
KCl std	2090	0	109	0	0	99.2	0
Mg std	4880	0	0	975	0	0	3900
TDS Synthetic sol	2450	648	15	38	40	14	1700
Mix A	335	62.5	12.5	12.5	12.5	11.9	223
Mix B	267	12.5	62.5	12.5	12.5	59.4	107.2
Mix C	337	12.5	12.5	12.5	62.5	11.9	224.8
Mix D	414	12.5	12.5	62.5	12.5	11.9	302.1
25 ppm	338	25	25	25	25	23.8	214.3

Models of the results indicates that chloride ions are the most toxic component included in total dissolved solid measurments. These results and further discussion are presented in more detail in Chapter 5.

Chapter 5

Discussion

5.1 *Comparison of Assay Procedures*

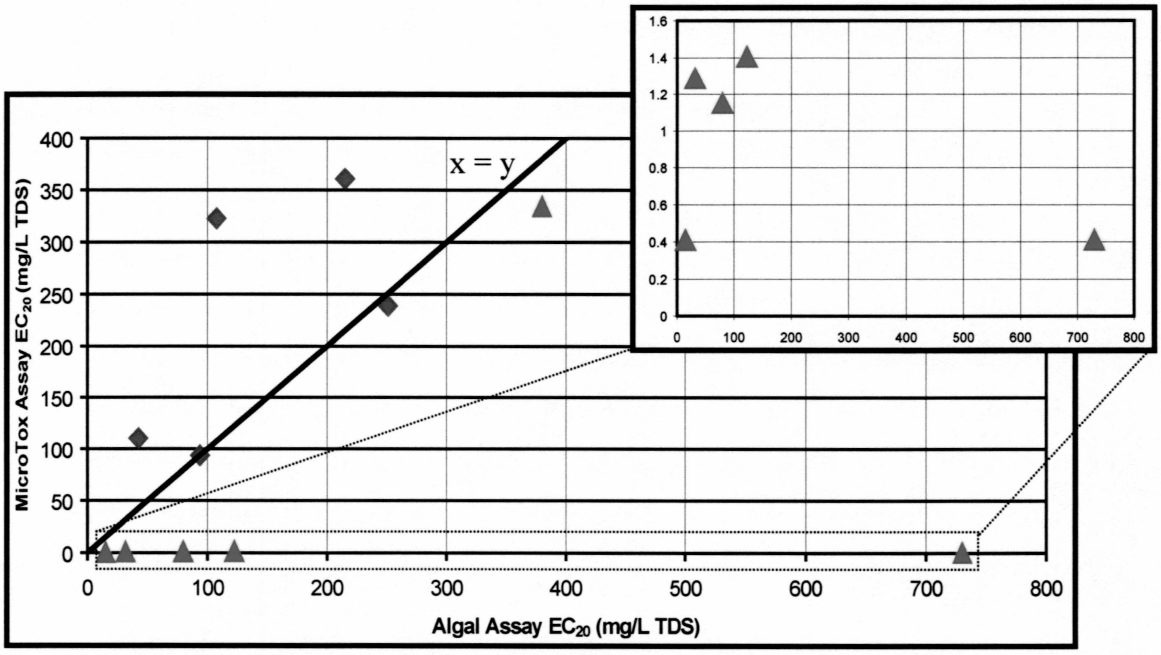


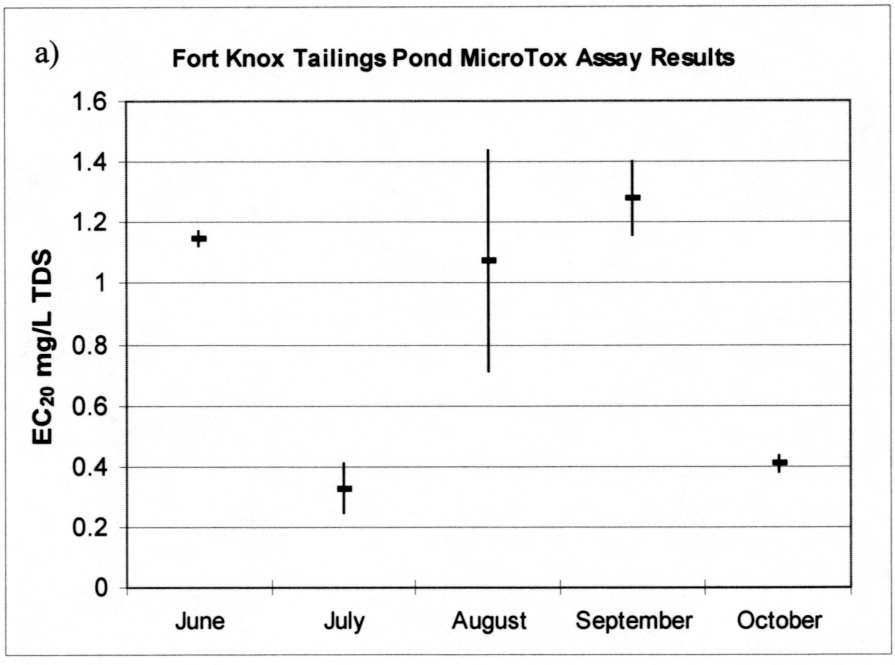
Figure 15. Algal versus MicroTox Assay results. Red Dog Station 140 samples denoted by diamonds, Fort Knox samples denoted by triangles. The inset image shows an expanded Y axis. Straight line indicates results of a 1:1 correlation.

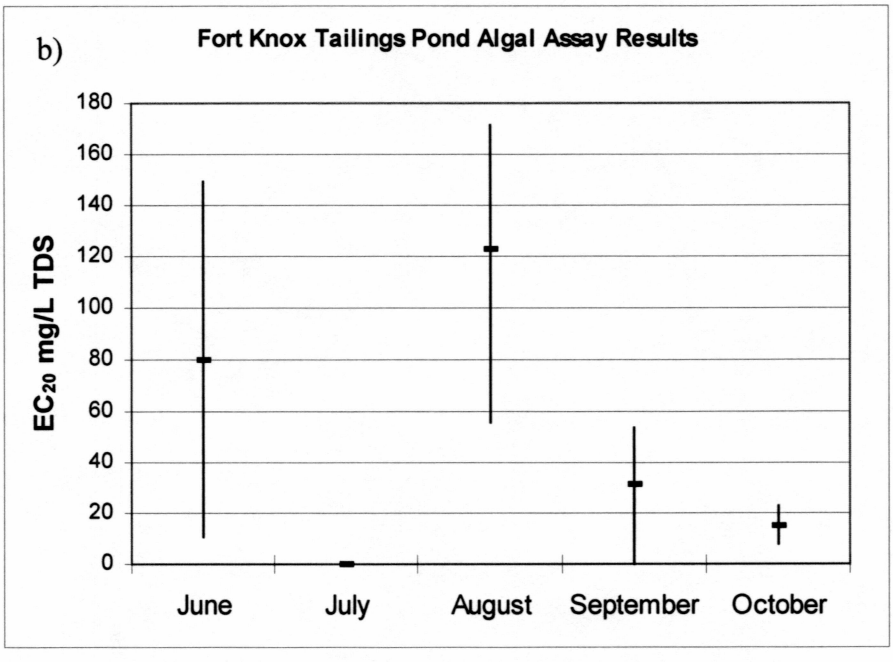
By inspection of the toxicity measurements of the field samples, it is apparent that the results are significantly different between the two assays. Toxicity data from the algal assay was plotted against the MicroTox® data to illustrate possible relationships between the measurements. Had the MicroTox® and algal assays shown equal sensitivities to the samples tested, all data points would have fallen on a line x = y. Points falling below the line represent samples demonstrating higher toxicity using the MicroTox® assay, while those above the line were more toxic according to the algal assay. Although there is a distinct relationship between the sampling site and the assay

sensitivity, the random scatter of the samples as a whole indicates that there is no direct relationship between the assay results. The Spearman rank order test also showed no obvious correlation between methods at a significance level of $P<0.05$.

5.2 *Accuracy and Precision*

One of the advantages of the MicroTox® over the algal assay is that it gives much more precise and reproducible results. Figures 16a and b illustrate the precision of multiple toxicity measurements using the MicroTox® and algal assays. The relative standard deviation for samples from the Fort Knox Tailings Pond was 65.9% using the algal assay, and 16.0% using the MicroTox®. Sample precision from Red Dog Station 140 was better, with a relative standard deviation of 52.5% for the algal assay and 6.55% for the MicroTox® assay. These standard deviations for the MicroTox® assay generally agree with those reported by DeZwart and Slooff (1983).





Figures 16(a) and (b). Precision of multiple measurements using the MicroTox® and algal assays. Horizontal lines indicate average toxicity values of repeat measurements, verticle lines indicate variance among repeat measurements. July tailings pond sample was non-toxic on the algal assay.

A scattering of data points and variability between replicates of identical concentration are common in bioassay tests. The MicroTox® assay is able to give excellent reproducibility and precision because of its highly standardized reagent organisms and method.

Often data variability is due to interspecies variability of the test organisms. Single-cell algal assays, however, have minimal differences between organisms, and are regularly used to avoid these complications. The source of inconsistancy in results using these assays usually stems from growth differences among sample containers, which does not necessarily reflect the organism's tolerance or sensitivity. These growth differences may be caused by errors in volume measurements, cell density measurements, or variability among cell growth due to light and temperature (Nyholm and Kallqvist, 1989). This inherent variability of algal assay procedures limits on the precision of results. This study indicates that these variations continue to be a problem, and limit the confidence of results from this assay.

5.3 *Dose Response Curves*

It was expected that the two assays would exhibit linear dose-response curves. Results from the MicroTox® assay did indeed demonstrate a linear relationship between effluent concentration and organism response, as seen in Figure 17. However, results from the algal assay often followed a non-linear response curve relationships, as illustrated in Figures 18 and 19.

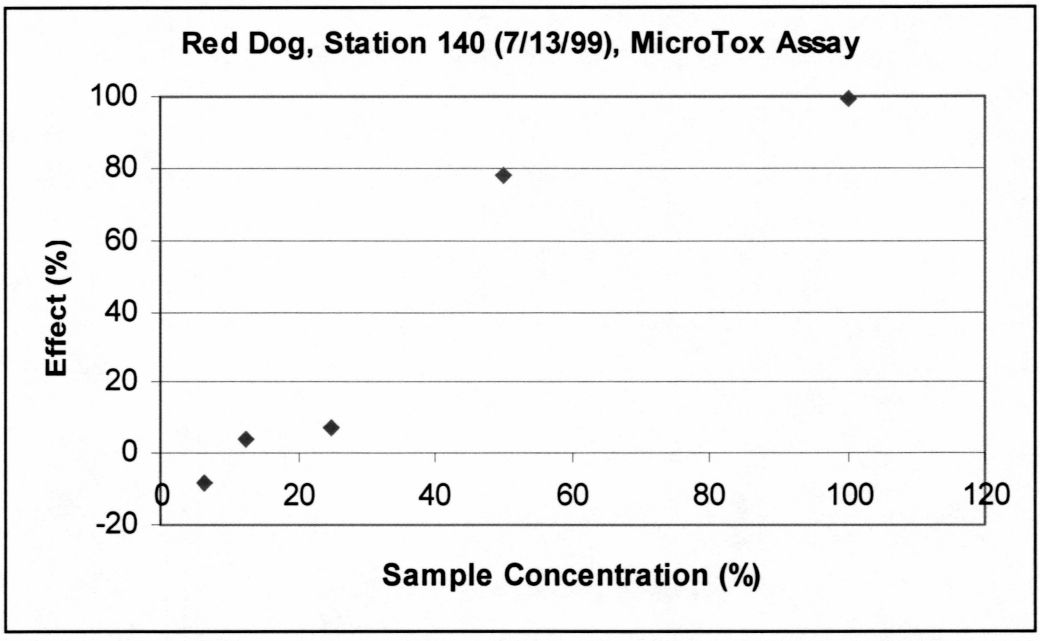


Figure 17. Linear dose-response curve as demonstrated by the MicroTox® assay.

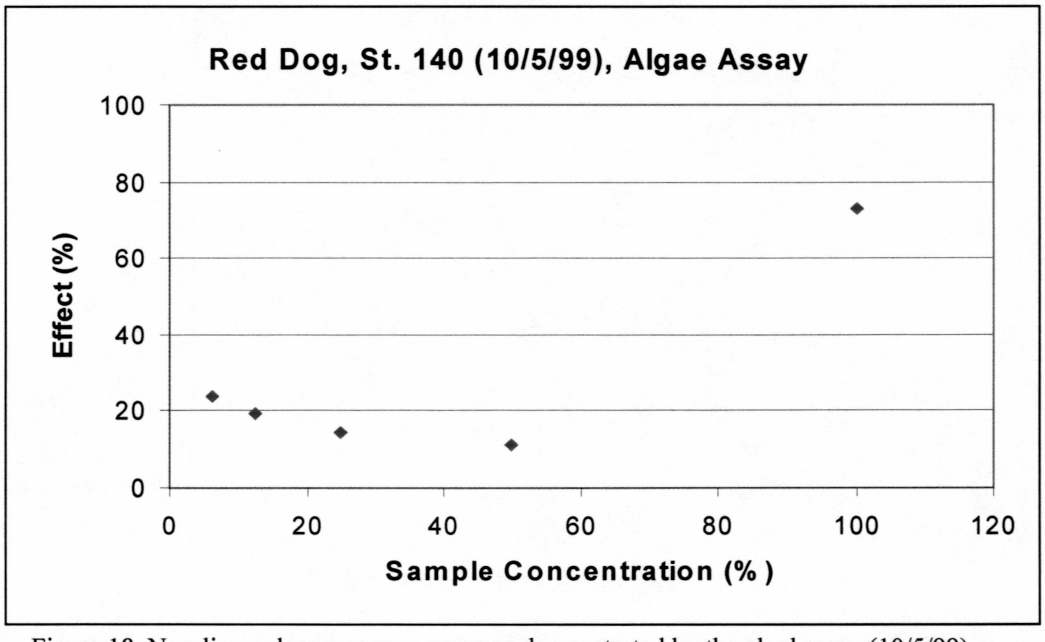


Figure 18. Non-linear dose-response curve as demonstrated by the algal assay (10/5/99).

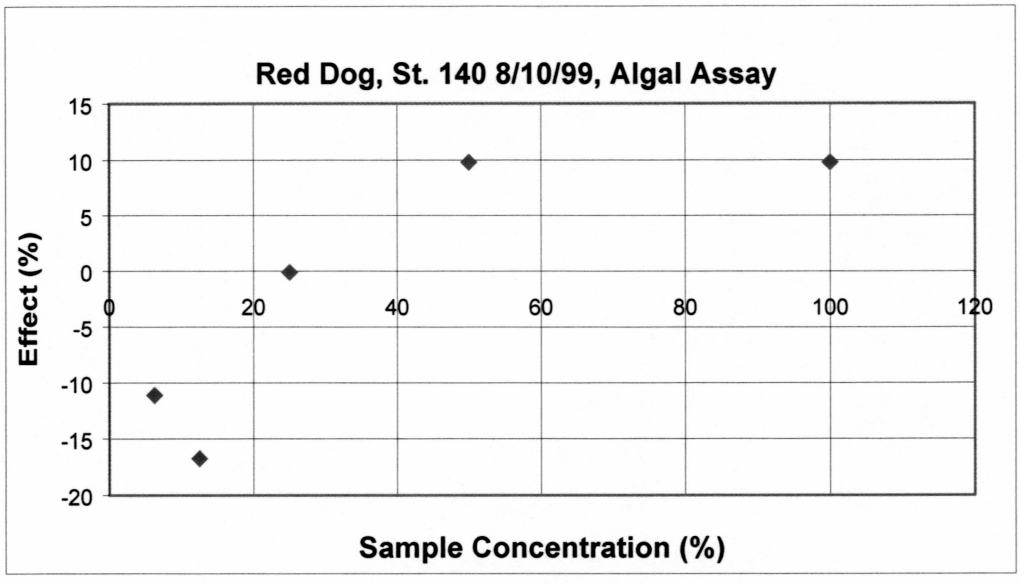


Figure 19. Non-lineaer dose response curve as demonstrated by the algal assay (8/10/99).

Many of the tested water samples elicited a stimulation in algal growth at lower concentrations, while inhibiting growth at higher concentrations. This behavior was seen using both the bacteria and algae, and was believed to be a result of outlying or variable data points. These results, however, appear to be common with particular algal assays. Nyholm and Kallqvist (1989) indicate in their algal assay review that often these assay dose-response curves are sigmoidal rather than linear. They observed that stimulatory growth responses sometimes occurred prior to growth inhibition, and hypothesized that this behavior was due to toxicant induced physiological stress or imbalance. There are no standard procedures to address this initial stimulation, and it is suggested that the initial data curve be ignored in EC calculations.

5.4 *MicroTox® Assay*

Standard Procedure guidelines for the MicroTox® chronic assay recommend that all control cuvette readings be between 90 and 110 units. Control light readings rarely reached 95 units, and typically varied between 92 and 78 units, even when measured at the maximum 26 hours. Due to budget limitations, the temperature control bath for the cuvettes, supplied by AZUR Environmental, was not purchased. Incubation of the cuvettes was done at room temperature, which remained between 20 and 22° C. This is below the method temperature of 27° C, which may have slightly decreased the luminescent values of the controls and samples. Despite this complication, the MicroTox® chronic assay gave highly reproducible results with far less laboratory work than the algal assay.

5.5 *Algal Assay*

Although each of these assays was chosen for its reputation for rapid results and little required knowledge of microbiology, the algal assay proved to be very time consuming and difficult to work with. Complications were encountered with the culturing of organisms, maintaining the survival of control cells, and achieving reproducible results.

Growing batch cultures of *S. capricornutum* was a much more delicate process than anticipated. Constant dividing of batches was necessary to maintain the growth of the cells, but growth rates remained highly variable, despite constant light and temperature conditions. On occasion, batches would become contaminated with bacteria,

from either the air, glassware, or media, and would be discarded. Manually aerating cultures daily with carbon dioxide increased the rate of cell growth.

As with batch cultures, the growth of control organisms during individual tests was also highly variable. Control cell densities would often increase considerably in the first 48 hours of the test period, and decrease in the last 24 hours. In order to accurately gauge the toxic effects of a compound, organisms must be dosed during the growth phase. Therefore, a decrease in growth of the control cells renders a test run unusable. The measured growth of the control cells must also be large enough to calculate toxicant results with some level of confidence. On occasion, cell growth of the controls was not adequate to run these calculations, and the samples were rerun.

Reproducibility between replicates within algal assay tests was often poor, with EC_{20} results sometimes differing by 100% or more. Samples rerun at later dates appeared to decrease in toxicity, although these observations may be variation in the assay, not a chemical change in the samples themselves. No appreciable change in toxicity was observed with occasional sample reruns using the MicroTox® assay.

The *S. capricornutum* bioassay, like most plant cell assays, involved growing organisms under non-optimum conditions. This condition is usually due to deficiencies in nutrient salts in the media, carbon dioxide, or light (Klaine and Ward, 1983). When using a nutrient poor medium, added ions will likely act as nutrients to the test organisms. Because this behavior was most often observed when testing individual ion solutions, it is believed that the test organisms were stimulated by the increase in these deficient nutrients. The concentration corresponding to maximum growth stimulation on each

dose-response curve may indicate the optimum concentration of that component for test organism growth.

Further complications in toxicity measurements on the algal assay stem from changes in sample pH during the duration of the testing. Maintaining a constant pH value is important, especially when samples include heavy metals and weak organic acids or bases. This is because the toxicity of these elements can change drastically with pH. There are a number of reactions by which assay samples may undergo changes in pH such as the uptake of CO_2 from the air. Algae require carbon for photosynthesis, and typically this source is dissolved carbon dioxide. When dissolved CO_2 is depleted, cells begin to consume dissolved bicarbonate by through the following net reaction:

$$HCO^-_3 \rightarrow CO_2 + OH^-$$

by which hydroxide ions are generated, leading to an increase in pH. Unless another nutrient or light becomes limiting, the pH will continue to rise until the bicarbonate is exhausted. Studies by Nyholm and Kallqvist (1989) indicate that static sample cultures undergo a significant pH increase in as little as 48 hours. In one series of experiments, the pH of these cultures increased from 7.5 to nearly 10 over a period of four days. To avoid these effects, it is has been suggested that test flasks or tubes be aerated or agitated during the course of an experiment. Both processes facilitate carbon dioxide transport into the solutions.

Toxicant studies on samples containing heavy metals are also subject to error due to adsorption. Certain ions and chemicals are capable of adsorbing to the surface of the algae biomass. Adsorption increases as the biomass increases, which decreases the

toxicant concentration in solution (Nyholm and Kallqvist, 1989). The result will be a toxicity value that underestimates the true toxicity value. This phenomenon has been demonstrated in studies with copper (Steemann, 1970), chromium (Wium-Andersen, 1974), and cadmium (Truhaut et al., 1980).

Test medium, as well, can influence the toxicity of many of the components of a solution (Peterson, 1984). The effect of limiting nutrients in medium was observed previously with the dilution experiments. In addition to major cation concentrations, hardness, pH, and the presence of chelators can influence the toxicity of a tested sample. For the most accurate toxicity values for a given water sample, Nyholm and Kallqvist (1989) recommend using site-specific natural water that has been filtered and enriched with nutrients.

5.6 *Multi-Component Analysis*

5.6.1 *Model of MicroTox® Results*

Principle component analysis was used to study the relationship between sample composition and the observed toxicity. *Unscrambler* (Trondheim, Norway), a multi-variant analysis software program, models relationships between independent and dependent variables based on significant variations within a data matrix. Plots are generated using principle components as axis, and variables are plotted along these axis in multidimensional space. More detailed information on multi-variant statistics and modeling can be found in Multivariate Analysis in Practice by Kim Esbensen *et al.*, (1994), and Beebe and Kowalski, (1987).

Concentrations of 26 chemical constituents, sampling month, and location were used as independent variables. Toxicity, as measured on each assay, was used as the dependent variable. The best model was produced using principle component least squares regression statistics, with the 26 chemical components as independent variables. The qualitative toxicity result of each assay was designated as the single dependent variable. Two significant principle components were identified, with the first PC accounting for 60% of the toxicity variation, and the second PC accounting for an additional 3%. Independent variables were weighted using inverse standard deviation, and the data was centered about the origin. The resulting models yield important information about both the samples and the bioassays.

General groupings of like samples are displayed in the bi-plot, Figure 20. These groupings indicate that each site has a distinctive chemical composition, which remained fairly constant throughout the duration of the sampling period. Loose sample clusters from the Fort Knox Tailings Pond, and Red Dog Stations 001, 140, and 9 are most evident. Many samples are tightly clustered about the axis, which indicates they have similar chemical composition. Alternatively, this clustering may also show that these samples are poorly represented by the model.

The proximity of plotted elements to sample clusters indicates a direct relationship between those ions and water from that site. For example, nickel, lead, and cadmium lie among samples from Station 140. This site contains very high levels of these metals, which were not present in appreciable amounts at any other site in this

study. Similarly, water from the Fort Knox Tailings Pond had very high chloride levels compared to other sites.

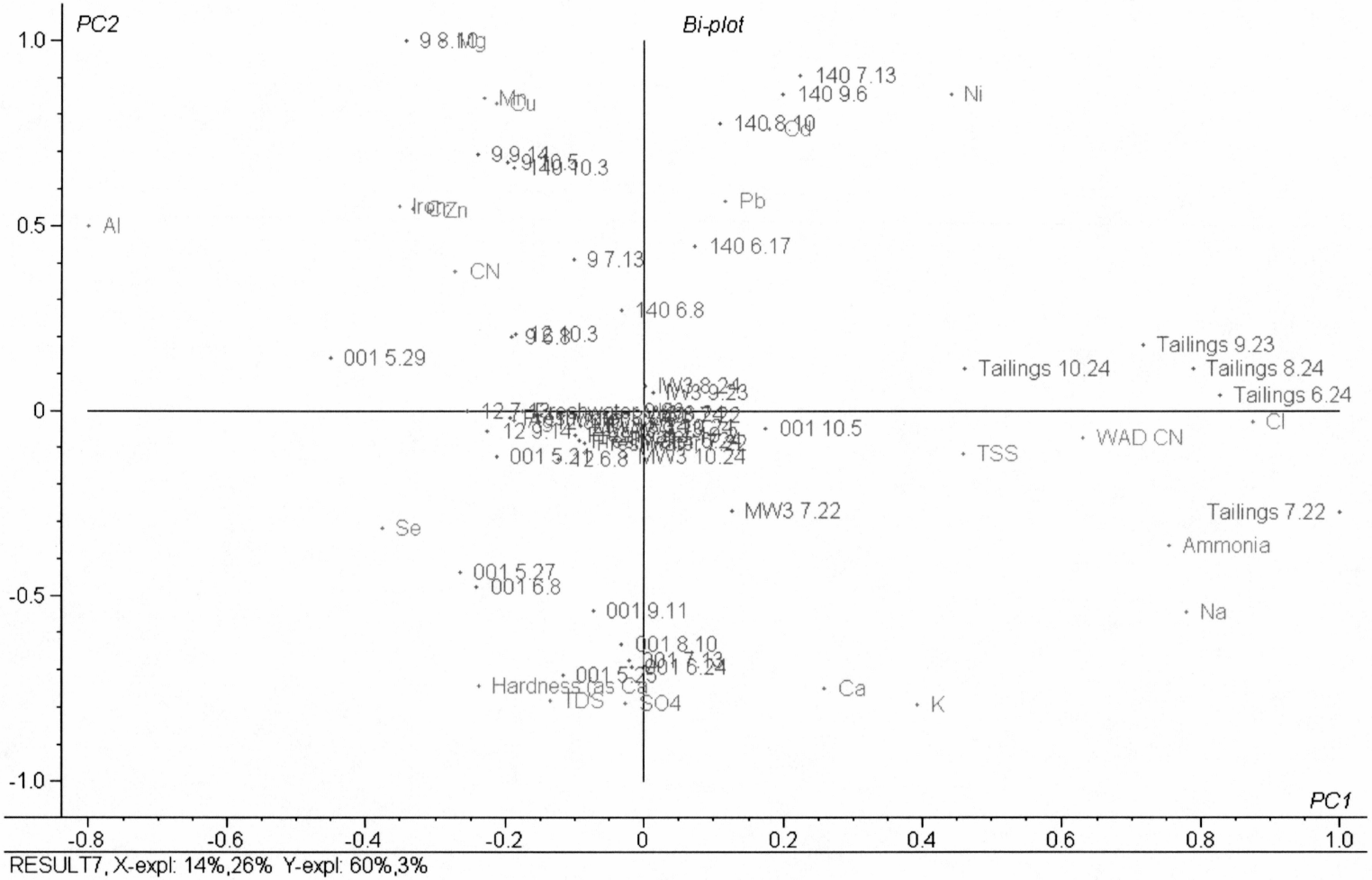


Figure 20. Bi-plot of field samples tested using the MicroTox®.

When interpreting results from the X loading weights and Y loading plot, factors lying farthest from the origin have the greatest significance in the model. Distance from the origin along the X axis indicates significance on the first PC, while distance from the origin along the Y axis indicates significance on the second PC. At the bottom of each plot is a percent value, which represents the variance in toxic behavior explained by each principle component. High explained variance indicates a good model, that is, one that explains most of the observed toxic behavior of the samples.

Principle component analysis results from the MicroTox® assay are illustrated in Figure 21. In this model, the primary components correlated with toxicity on the MicroTox® assay are chloride, ammonia, and nickel. To a lesser degree, cyanide, sodium, and cadmium correlate with toxicity. It should be observed that cyanide analysis was done on few sample sites. Therefore, these results may not be a complete representation of the effects of cyanide on the MicroTox® assay.

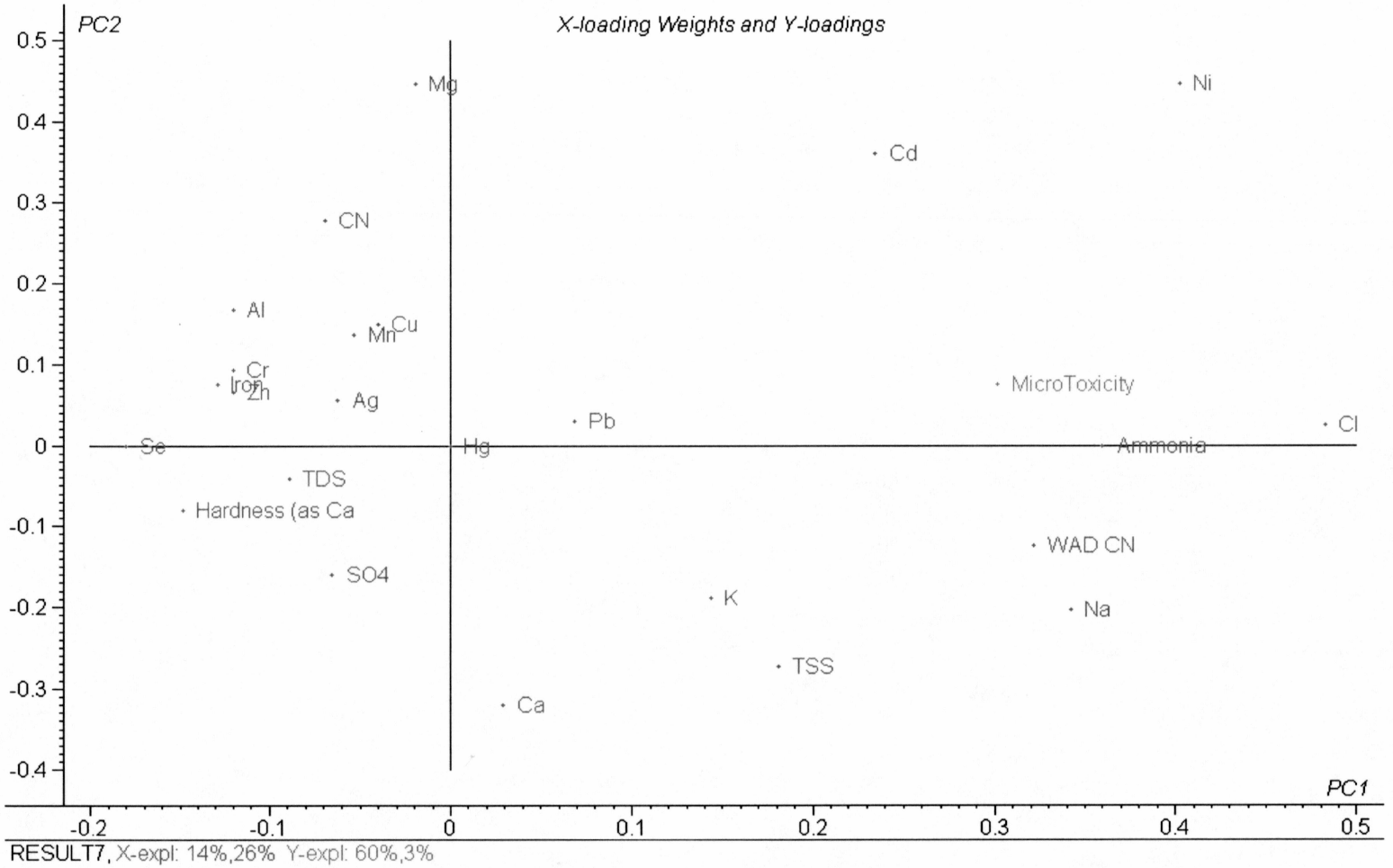


Figure 21. X loading weights and Y loading plot of samples tested on the MircoTox® assay.

Previous studies of ammonia in industrial effluent have found that ammonia is primarily toxic to aquatic organisms in the un-ionized form as "free ammonia", NH_3 (Hinwood and McCormick, 1987). As NH_4^+, the ionized form, the ammonium ion is much less toxic. These two forms are present in aqueous solutions in equilibrium, with their concentrations dependent upon pH, temperature, and salinity of the water.

5.6.2 *Model of Algal Assay Results*

A biplot model (Figure 22) and an X-loading weight and Y-loadings plot (Figure 23) describing sample results using the algal assay, using the same chemical constituents as independent variables, and a qualitative measure of toxicity as the dependent variable, were also constructed. Again, two significant principle components were identified, the first PC accounting for 43% of the toxicity variation, and the sencond for and additional 18%. PC1 is most strongly correlated to cadmium, as demonstrated by the magnitude of the cadmium point on the X axis.

Modeling analysis of *S. capricornutum* results indicates that this assay is most sensitive to cadmium and chloride, and to a lesser degree, nickel and sodium. The results of this study are consistant with those of a previous study which demonstrated a relationship between the toxicity of produced waters and the chloride content (Mount et al.,1992).

The toxicity of cadmium to algal assays has also been documented. A 1990 study by Versteeg found that cadmium produces an EC_{50} of 0.13 mg/L on the *Selenastrum capricornutum* assay, while Radix et al.(1999) found that the MicroTox® assay produces

an EC_{50} of 0.214mg/L. These values indicate that *S. capricornutum* is more sensitive to samples high in cadmium. These results explain the sensitivity of the algal assay to samples from Red Dog Station 140. These samples contained cadmium levels of 0.04 to 0.012mg/L, which were much higher than any other samples tested in this study.

Toxic effects of cadmium on *S. capricornutum* are highly dependent upon sample pH, and have been shown to increase up to eight times for every increase in pH unit (Peterson, 1991). Changing pH levels during the assay incubation period, as referenced by Nilhom and Kallqvist (1989), may have been a factor in the effects of cadmium on the toxicity of the Station 140 samples.

Cyanide and lead, two highly toxic components appear to be inversely correlated to toxicity. These results are misleading, however. Cyanide analysis was not done on all sample sites from Red Dog Mine, and was present at or below method detection limits (0.01mg/L) for most of those sites tested at Red Dog and Fort Knox Mines. Lead, as well, was at or below detection limits (0.002mg/L) for most sites at Red Dog and Fort Knox Mines.

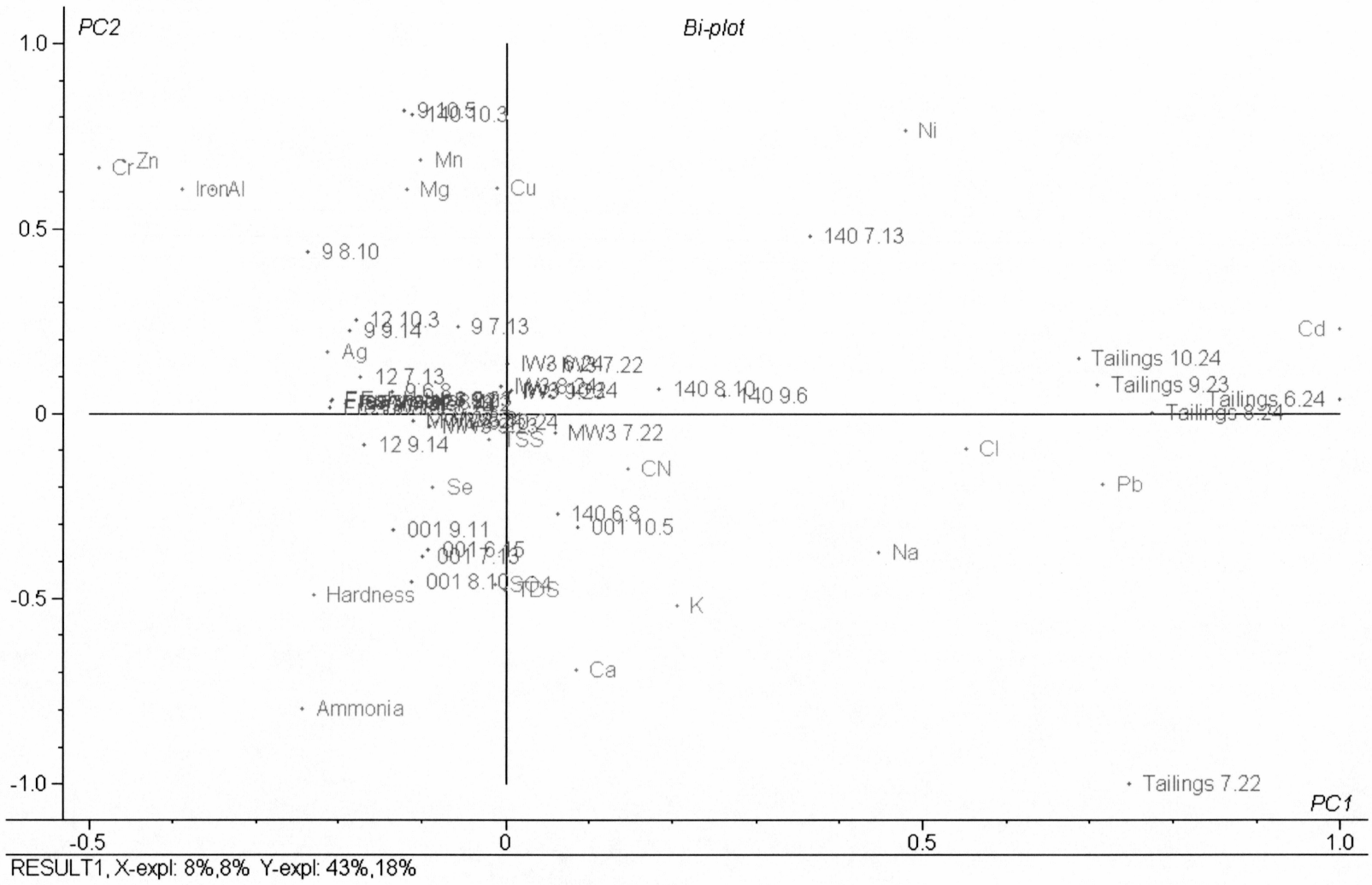


Figure 22. Bi-plot of field samples tested using the algal assay.

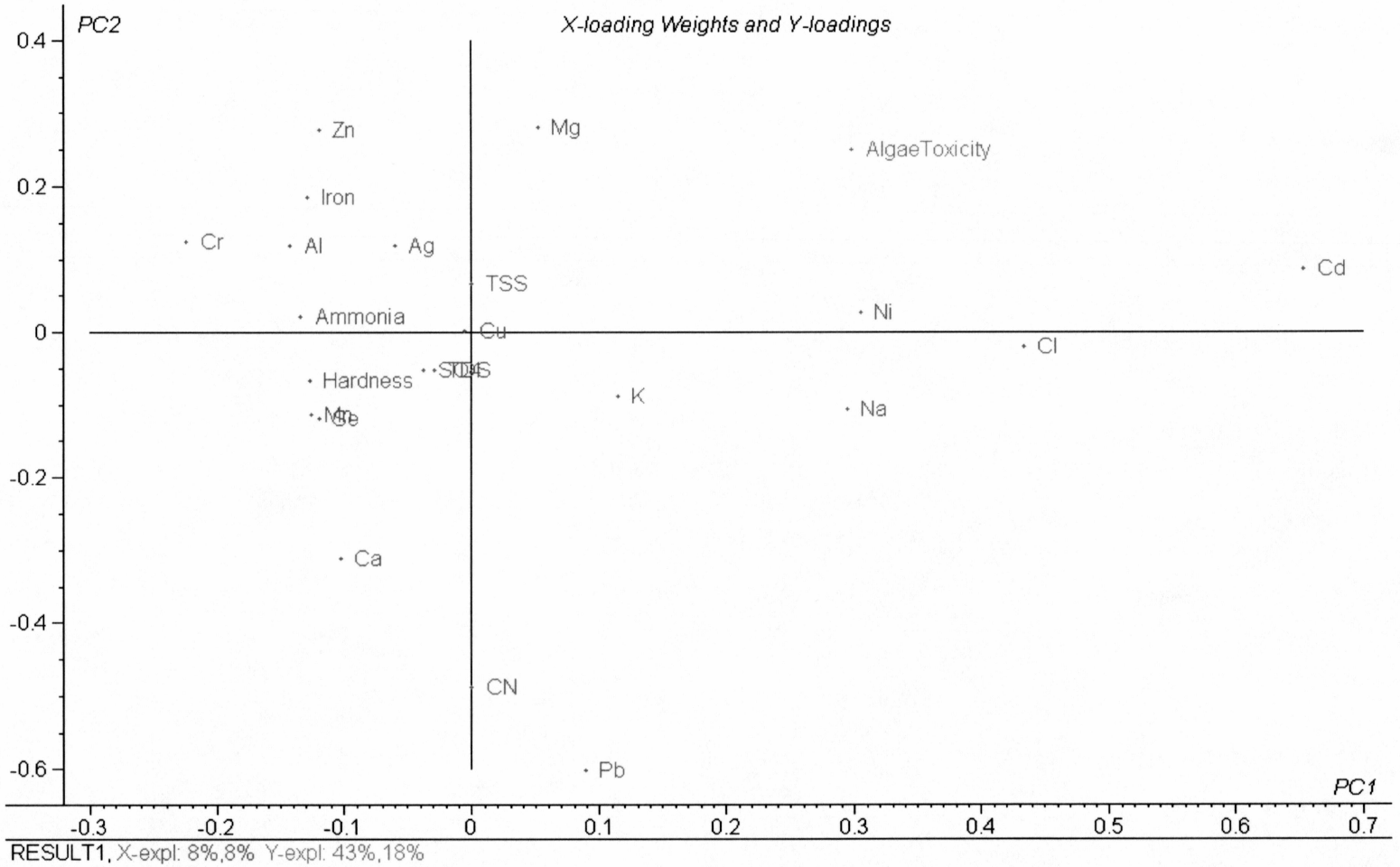


Figure 23. X-loading weights and Y-loadings plot of field samples tested using the Algal Assay.

5.6.3 *Model of Individual Salt Standards*

Another model was built using the toxicity results from the individual and mixed salt standards, and the 2500 ppm synthetic TDS solution. For this model, the dependent variables used were the concentrations of the six cations and anions. Instead of using a qualitative measure of toxicity for this model, the EC_{20} value (in ppm TDS) was used as the dependent variable. Since the toxicity and EC_{20} of a sample are inversely related, the model must be interpreted slightly differently than the previous field sample models. On these plots, the proximity of ion species to the dependent variable indicates a benign component. Those components plotted opposite the EC_{20} value are most responsible for the toxicity of the sample.

Results of the X loading weights and Y loadings plot, Figure 24, indicates that toxicity measured on the MicroTox® assay is most closely correlated to the presence of chloride and potassium. Calcium and sodium apprear inversely related to toxicity. Magnesium has a high loading on the PC2 axis, which accounts for 28% of the observed toxic behavior.

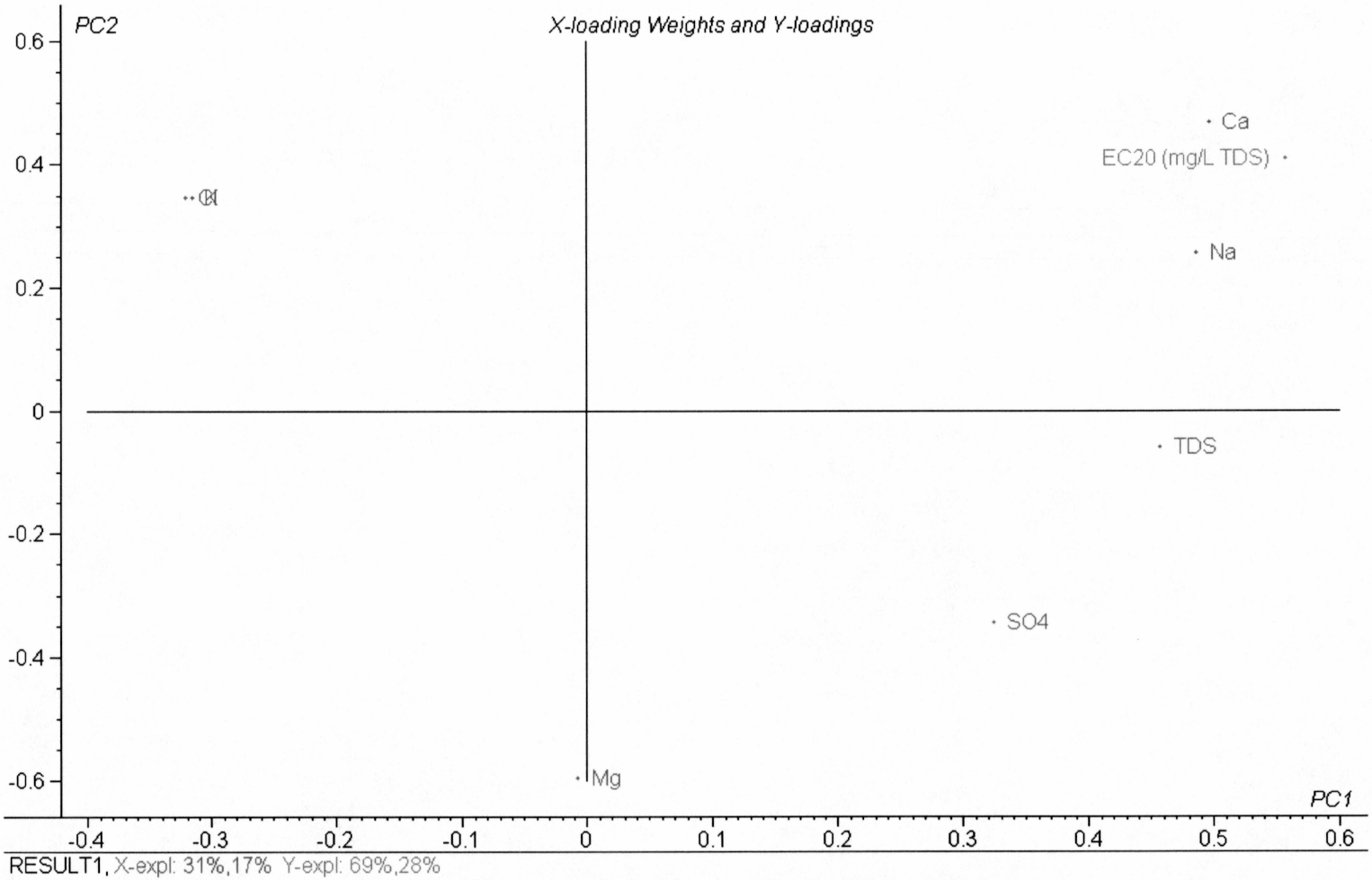


Figure 24. X-loading weights and Y-loadings of TDS ion standards using the MicroTox® Assay.

5.6.4 *Design Expert Analysis*

These results were also analyzed using *Design Expert* (Milwaukee, Wisconsin). This program uses a variety of plots to analyze the significance of factors and results from designed experiments. Normal and half normal plots of results are used to distinguish significant variables and outliers. Normal plots graph normal percent probability versus effect. If none of the variables are significant, they will fall on a straight line about the zero on the Y-axis. The normal plot of the original results, Figure 25, reveals little information as to the significance of the variables.

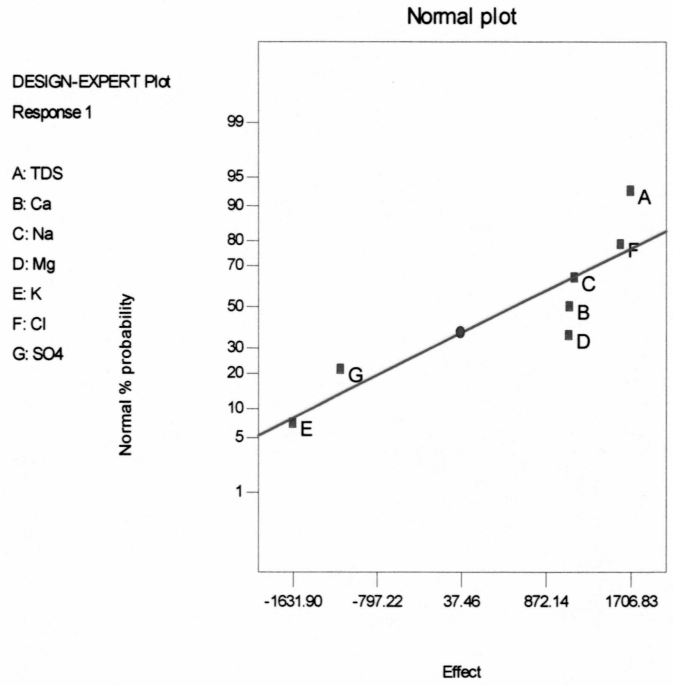


Figure 25. Normal plot of TDS ion standards tested using the MicroTox®.

None of the factors deviates significantly from the trend line.

The response values in mg/L TDS encompased a broad numerical range. In order to ensure homogenized standard deviations, these values were mathmatically transformed

using $\log_{10}$. Results with this transformed data is shown in Figure 26. These plots indicates factors K, Cl and TDS may be significant in determining toxicity responses.

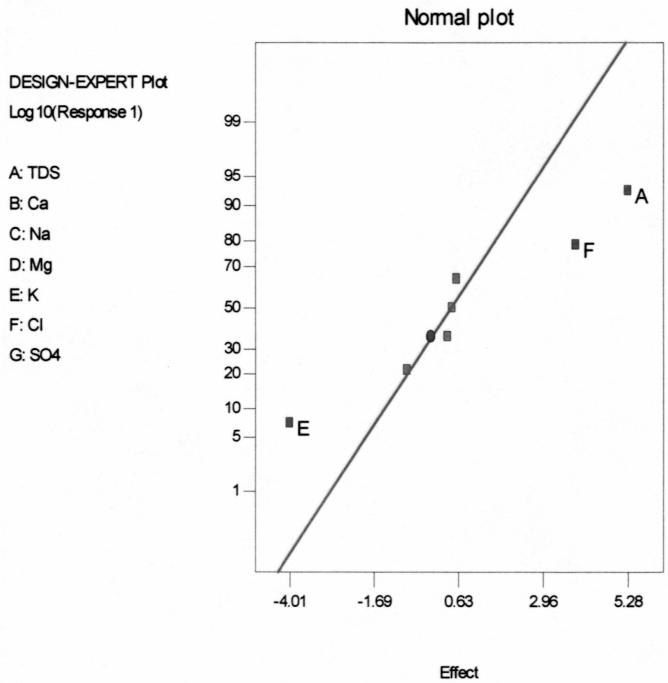


Figure 26. Normal plot of $\log_{10}$(response) ofTDS ion standards tested using the MicroTox®. Toxicity responses have been transformed using $\log_{10}$. Factors B (Ca), C (Na), D (Mg), and G (SO_4^{2-}) are insignificant and not labeled.

This generally agrees with the previous principle component analysis on *Unscrambler,* although *Unscrambler* did not identify TDS concentration as a significant factor.

These results for chloride and potassium were demonstrated earlier by the MicroTox® assay. In addition to the recognized effects of chloride and potassium, TDS concentration also demonstrates a significant influence. These results differ from those of the field samples, in which data analysis did not show TDS to be a major contributing factor of observed toxicity on the bioassays. TDS concentrations in field samples were

lower, and sometimes much lower than those of some of the laboratory standards. Field samples contained TDS concentrations between 94 and 870 mg/L, while the standards varied between 267 and 4880 mg/L. Only at these high levels, and without the presence of other trace components, does TDS becomes a driving toxic factor.

Chapter 6

Conclusions and Suggestions for Further Study

This study answered several initial questions regarding the effects of effluent and TDS released to freshwater systems. The first being that toxic effects of total dissolved solids in discharged effluent cannot be predicted by a non site-specific synthetic TDS solution. The concentrations of individual ions within the TDS classification are a result of multiple chemical and physical factors unique to each environmental system. These individual ions produce various toxic responses from assay organisms. Therefore, the sensitivity of freshwater organisms is a result of additive, synergistic, or antagonistic effects of these ions. Standards of dissolved mass, without regard to species concentration, are unable to predict the toxicity of waters with similar TDS measures.

Composition of the receiving water will influence the toxicity of discharged effluent, as demonstrated by the mixing experiments. Thus, only a solution made with site-specific water, or water having identical ionic species composition will adequately predict the toxic effects of effluent to that natural system.

The second conclusion drawn from this work is that the toxicity of mine effluent cannot be predicted from the concentration of TDS ions alone. Typically mine waters contain trace metals and other chemical species which can produce toxic responses in freshwater biota when present at parts per million levels. TDS ions have demonstrated little toxic effects to bacteria and algae at these concentrations. In mine effluent, the effects of increased concentrations of cadmium, chromium, lead, and copper appear to

pose a much greater risk to these receiving systems than the elevated levels of metal salts. In fact, the increased concentration of available ligands, such as calcium and sulfate, may offer a degree of protection and nutrition to organisms in these systems.

As mentioned in Chapter 1, multispecies analysis is an important part of toxicity testing. It provides a more complete and accurate picture of the effects of released effluent into freshwater systems. Results from this work demonstrate that the MicroTox® assay is a useful tool for the initial screening of effluent. Results are easily reproducable, and relatively inexpensive. The general lack of correlation between results of the two assays indicates that the MicroTox® may not be suitable as a replacement for the algal assay for overall toxicity assessment. Future assay work may involve the investigation of other assays that may produces results similar to that of the MicroTox®.

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Appendix A

Chemical Data from Field Samples

Sample	Al µg/L	Ammonia (mg/L)	Cd (µg/L)	Ca (mg/L)	Cl (mg/L)	Cr (µg/L)
140 6.8	174	0.00	14.5	12.1	0.40	0.00
140 7.13	35	0.00	40.8	52.2	0.40	0.00
140 8.10	58	0.00	23.9	39.5	0.40	1.50
140 9.14	224	0.00	19.8	44.2	0.40	2.00
140 10.3				51.1	0.60	
001 6.8		3.67	0.75		1.89	0.00
001 7.13	31	8.28	0.70	795	2.21	0.50
001 8.10	0	8.58	1.03	835	2.31	0.00
001 9.14	28	8.20	0.97	752		0.00
001 10.5				702	1.97	
12 6.8	95	0.00	0.00	31.9	0.40	0.00
12 7.13	37	0.00	0.07	105	0.40	3.40
12 8.10	0	0.00	0.11	80.7	0.40	2.90
12 9.14	23	0.00	0.20	84.6	0.80	3.60
12 10.3				96.6	0.60	
9 6.8	290	0.00	0.85	18.4	0.50	1.00
9 7.13	93	0.00	1.33	63.6	0.30	1.30
9 8.10	447	0.00	4.98	50	0.40	3.70
9 9.14	287	0.00	2.58	51.2	0.50	3.70
9 10.5				69.9	0.50	
Tailings 6.24		33.4	0.00	133	36.8	0.00
IW3 6.24		0.00	0.00	73.1	6.19	0.00
MW3 6.24		0.20	0.00	42.4		0.00
Freshwater 6.24		0.12	0.00	17.3	0.50	0.00
Tailings 7.22		25.0	0.00	1030	33.3	0.00
IW3 7.22		0.00	0.00	72.5	7.89	0.00
MW3 7.22		0.00	0.00	36.4	0.96	0.00
Freshwater 7.22		0.30	0.00	18.3	0.52	0.00
Tailings 8.24				111	29.1	
IW3 8.24				59.8	5.18	
MW3 8.24				39.7	1.08	
Freshwater 8.24				19.7	0.53	
Tailings 9.23				93.2	29.6	
IW3 9.23				54.9	5.15	
MW3 9.23				38.9	1.51	
Freshwater 9.23				18.6	1.01	
Tailings 10.24		17.50	0	90.4	28.2	0.00
IW3 10.24		0.06	0	52.7	5.03	0.00
MW3 10.24		0.00	0	42.9	1.48	0.00
Freshwater 10.24		0.05	0	16.7	1.04	0.00

Sample	Cu (µg/L)	CN (µg/L)	WAD CN (µg/L)	Hardness (mg/L)	Fe (µg/L)
140 6.8	3.0			55.8	797
140 7.13	2.0			232	0
140 8.10	3.0			186	110
140 9.14	8.0			204	578
140 10.3				242	
001 6.8	0.0	0.006	0.00	1890	
001 7.13	0.0	0.004	0.00	2210	84.0
001 8.10	0.0	0.008	0.00	2310	0.0
001 9.14	0.0	0.006	0.00	2100	99.0
001 10.5				79.0	
12 6.8	0.0			96.9	161
12 7.13	0.0			318	23.0
12 8.10	0.0			250	24.0
12 9.14	0.0			256	32.0
12 10.3				294	
9 6.8	3.0			73.9	824
9 7.13	1.0			257	237
9 8.10	10			209	3250
9 9.14	5.0			207	1520
9 10.5				293	
Tailings 6.24	0.28	0.030	0.01	325	0.33
IW3 6.24	0.00	0.000	0.00	188	0.05
MW3 6.24	0.00	0.000	0.00	106	11.12
Freshwater 6.24	0.00	0.000	0.00	55.0	2.68
Tailings 7.22	0.45	0.220	0.18	295	0.59
IW3 7.22	0.00	0.000	0.00	186	0
MW3 7.22	0.00	0.000	0.00	89.0	0
Freshwater 7.22	0.00	0.000	0.00	45.0	3.78
Tailings 8.24					
IW3 8.24					
MW3 8.24					
Freshwater 8.24					
Tailings 9.23					
IW3 9.23					
MW3 9.23					
Freshwater 9.23					
Tailings 10.24	0.17	0.03	0.01	231	0.18
IW3 10.24	0	0	0	137	
MW3 10.24	0	0	0	103	0
Freshwater 10.24	0	0	0	48	1.6

Sample	Cu (µg/L)	CN (µg/L)	WAD CN (µg/L)	Hardness (mg/L)	Fe (µg/L)
140 6.8	3.0			55.8	797
140 7.13	2.0			232	0
140 8.10	3.0			186	110
140 9.14	8.0			204	578
140 10.3				242	
001 6.8	0.0	0.006	0.00	1890	
001 7.13	0.0	0.004	0.00	2210	84.0
001 8.10	0.0	0.008	0.00	2310	0.0
001 9.14	0.0	0.006	0.00	2100	99.0
001 10.5				79.0	
12 6.8	0.0			96.9	161
12 7.13	0.0			318	23.0
12 8.10	0.0			250	24.0
12 9.14	0.0			256	32.0
12 10.3				294	
9 6.8	3.0			73.9	824
9 7.13	1.0			257	237
9 8.10	10			209	3250
9 9.14	5.0			207	1520
9 10.5				293	
Tailings 6.24	0.28	0.030	0.01	325	0.33
IW3 6.24	0.00	0.000	0.00	188	0.05
MW3 6.24	0.00	0.000	0.00	106	11.12
Freshwater 6.24	0.00	0.000	0.00	55.0	2.68
Tailings 7.22	0.45	0.220	0.18	295	0.59
IW3 7.22	0.00	0.000	0.00	186	0
MW3 7.22	0.00	0.000	0.00	89.0	0
Freshwater 7.22	0.00	0.000	0.00	45.0	3.78
Tailings 8.24					
IW3 8.24					
MW3 8.24					
Freshwater 8.24					
Tailings 9.23					
IW3 9.23					
MW3 9.23					
Freshwater 9.23					
Tailings 10.24	0.17	0.03	0.01	231	0.18
IW3 10.24	0	0	0	137	
MW3 10.24	0	0	0	103	0
Freshwater 10.24	0	0	0	48	1.6

Sample	Pb (µg/L)	Mg (µg/L)	Mn (µg/L)	Hg (µg/L)	Ni (µg/L)	K (mg/L)
140 6.8	43.8	6.23	145.0		15	0
140 7.13	12.5	24.80	49.6		48	0
140 8.10	31.0	21.20	305.0		45.7	0
140 9.14	79.0	22.80	269.0		45	0
140 10.3		27.80				0
001 6.8	0.6	7.00	7.00		0	
001 7.13	0.72	2.00	2.00	0	2	33
001 8.10	1.27	5.80	5.80	0	2	27
001 9.14	0.88		8.80	0	2	31
001 10.5		17.90				3.56
12 6.8	0.37	4.20	14.0		7	0
12 7.13	0.12	13.40	5.00		2	0
12 8.10	0	11.70	8.50		9.6	0
12 9.14	0	10.90	8.70		8	0
12 10.3		12.80				0
9 6.8	1.05	6.81	274		20	0
9 7.13	0	23.90	239		33	0
9 8.10	2.12	20.30	608		55.8	0
9 9.14	0.75	19.20	517		47	0
9 10.5		28.80				0
Tailings 6.24	0	3.30	0.04	0		19.5
IW3 6.24	0	8.70	0.34	0		1.3
MW3 6.24	0.015	5.61	0.22	0		2.43
Freshwater 6.24	0	4.70	0.44	0		1.3
Tailings 7.22	0	3.30	0.05	0		19.3
IW3 7.22	0	8.00	0.30	0		1.2
MW3 7.22	0	3.40	0.09	0		0.8
Freshwater 7.22	0	4.60	0.62	0		1
Tailings 8.24		3.00				19.3
IW3 8.24		6.60				1.2
MW3 8.24		4.10				1.3
Freshwater 8.24		4.10				1.1
Tailings 9.23		3.27				13.3
IW3 9.23		6.15				2.25
MW3 9.23		3.74				2.25
Freshwater 9.23		4.73				2.25
Tailings 10.24	0	5.61	0.10	0		11.6
IW3 10.24	0	6.32	0.15	0		2.25
MW3 10.24	0	4.29	0.13	0		2.25
Freshwater 10.24	0	4.38	0.17	0		2.25

Sample	Se (μg/L)	Ag (μg/L)	Na (mg/L)	TDS (mg/L)	TSS (mg/L)
140 6.8	0	0	1.48	94	
140 7.13	0	0	5.95	356	
140 8.10	0	0	4.68	251	
140 9.14	2	0	4.64	323	
140 10.3			5.85	361	
001 6.8	3			2990	14
001 7.13	0	0	56.5	3240	0
001 8.10	0	0	41.5	3220	6
001 9.14	4	0	54.0	3310	0
001 10.5			69.0		
12 6.8	0	0	0.11	133	
12 7.13	0	0.05	4.06	396	
12 8.10	0	0	2.73	323	
12 9.14	3	0	2.40	362	
12 10.3			3.05	370	
9 6.8	0	0	1.09	117	
9 7.13	0	0	4.74	28	
9 8.10	0	0	3.40	274	
9 9.14	1	0	3.24	335	
9 10.5			4.94	396	
Tailings 6.24	0	0	97.3	870	28
IW3 6.24	0	0	24.9	380	0
MW3 6.24	0	0	26.2	203	149
Freshwater 6.24	0	0	3.20	98	5
Tailings 7.22	0.006	0	91.3	730	35
IW3 7.22	0	0	28.8	358	0
MW3 7.22	0	0	59.4	181	38
Freshwater 7.22	0	0	3.40	114	7
Tailings 8.24			79.8	650	
IW3 8.24			28	294	
MW3 8.24			23	208	
Freshwater 8.24			3.30	98	
Tailings 9.23			69.6	558	
IW3 9.23			29.2	289	
MW3 9.23			24.4	196	
Freshwater 9.23			3.94	96.3	
Tailings 10.24	0	0	70.9	578	0
IW3 10.24	0	0	29.8	290	0
MW3 10.24	0	0	26.3	231	8
Freshwater 10.24	0	0	3.67	109	0

Sample	SO4 (mg/L)	Zn (µg/L)
140 6.8	49	2.07
140 7.13	213	7.79
140 8.10	148	3.38
140 9.14	170	2.55
140 10.3	211	
001 6.8	1820	49
001 7.13	2140	42
001 8.10	1940	71
001 9.14	2330	78
001 10.5		
12 6.8	39.5	10
12 7.13	162	3.3
12 8.10	123	10
12 9.14	126	12
12 10.3	155	
9 6.8	30.6	254
9 7.13	167	367
9 8.10	128	1270
9 9.14	122	654
9 10.5	185	
Tailings 6.24	408	0.01
IW3 6.24	132	0.02
MW3 6.24	56	1.88
Freshwater 6.24	16.3	0.05
Tailings 7.22	356	0
IW3 7.22	144	0.01
MW3 7.22	59.4	0.11
Freshwater 7.22	14.7	0.03
Tailings 8.24	291	
IW3 8.24	113	
MW3 8.24	57	
Freshwater 8.24	13.3	
Tailings 9.23	279	
IW3 9.23	121	
MW3 9.23	65.8	
Freshwater 9.23	18.1	
Tailings 10.24	302	0.01
IW3 10.24	128	0.03
MW3 10.24	81.6	0.11
Freshwater 10.24	16.9	0.03